IDENTIFICATION AND CHARACTERIZATION OF PROTEIN KINASE CK2 AS A NOVEL INTERACTING PROTEIN OF NEURONAL CDK5 KINASE AND ITS FUNCTIONAL ROLE IN MICROTUBULE DYNAMICS

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Table of Contents

Abbreviations

a.a.	amino acid
AD	Alzheimer's disease
APP	amyloid precursor protein
ATP	adenosine triphosphate
***c*-abl**	*c*-Abelson
CAK	Cdk activating kinase
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
Cdk	cyclin-dependent kinase
Cdk5	cyclin-dependent kinase 5
cDNA	complementary deoxyribonucleic acid
CK1	casein kinase 1
CK2	casein kinase 2
CKI	Cdk inhibitor
CNS	central nervous system
cpm	counts per minute
DARPP-32	dopamine- and cAMP-regulated phosphoprotein, M_r 32 kDa
dATP	deoxy-adenosine triphosphate
dbpA	DNA-binding protein A
DEAE	diethylamino-ethylcellulose
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	1,4-dithiothreitol

ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetra acetic acid
EGTA	ethyleneglycoltetra acetic acid
ER	endoplasmic reticulum
FAK	focal adhesion kinase
FCS	fetal calf serum
FPLC	fast protein liquid chromatography
GSH	glutathione
GSK-3	glycogen synthase kinase-3
GST	glutathione-*S*-transferase
GTP	guanosine 5'-triphosphate
HA	hemagglutinin
Hepes	N-(2-hydroxyethyl)piperazine-N'-(1-ethane sulphonic acid)
hr	hour
HSP-70	heat shock protein 70
INK4	inhibitor of Cdk4
IPTG	isopropyl-1-thio-β-galactopyranoside
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
KIP	kinase inhibitor protein
LB	Luria-Bertani medium
M	molar
MAP	microtubule-associated protein
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2

MEK	mitogen-activated protein kinase kinase
min	minute
ml	milliliter
MOPS	4-morpholinepropanesulfonic acid
M_r	molecular mass
NFH	heavy chain of neurofilament protein
NFM	intermediate chain of neurofilament protein
NGF	nerve growth factor
nickel-NTA	nickel nitrilotriacetic acid
NLS	nuclear localization signal
NMDA	*N*-methyl-*D*-aspartate
NP-40	nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PAK	p21-activated protein kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI3-K	phosphatidylinositol 3'-kinase
PIPES	piperazine-N,N'-bis-(2-ethanesulfonic acid)
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PMSF	phenylmethylsulphonyl fluoride
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
pRb	retinoblastoma protein
PVDF	polyvinylidene difluoride

rpm	revolutions per minute
RNAi	ribonucleic acid interference
RT	reverse transcription
SDS	sodium dodecyl sulfate
siRNA	small interfering ribonucleic acid
sec	second
Tris	2-amino-2(hydroxymethy)-1-3-propanediol
UV	ultraviolet

Abstract

Neuronal cyclin-dependent kinase 5 (Cdk5) has been shown to play an important role in a variety of cellular processes, including neuronal cell differentiation, apoptosis, neuron migration and synaptic plasticity (Dhavan and Tsai, 2001; Lim *et al*., 2003). The active kinase consists of a catalytic subunit, Cdk5, and a regulatory subunit, p35 or p25, which are expressed primarily in neurons. Little is known about the regulation of Cdk5/p35 kinase turnover/activity, apart from the fact that it is degraded through the ubiquitin proteosome pathway (Patrick *et al*., 1998). We were interested to explore the regulation of neuronal Cdk5 activity. To achieve this, p35 and its fragments were made as GST-tagged fusion proteins and utilized in biochemical affinity purification attempts to isolate novel p35-binding proteins. We have also employed a co-immunoprecipitation approach in our search for novel interacting proteins. Using the former approach, the catalytic α subunit of protein kinase CK2 (formerly known as casein kinase 2) was isolated from rat brain extracts. The direct associations of CK2 with p35, as well as with Cdk5, were demonstrated and the CK2-binding sites of p35 were delineated. We showed that CK2 exhibited a strong inhibition on Cdk5 activation by p35 *in vitro* and *in vivo*. Cdk5 inhibition however is not associated with CK2 kinase function, since a kinase-dead CK2 mutant displayed a similar level of Cdk5 inhibitory activity as the wild-type protein. Interestingly, further analysis revealed that CK2 acts by blocking the formation of a complex between Cdk5 and p35. Hence, CK2 exerts a direct negative effect on Cdk5 activation by p35 through its physical interaction with p35.

Regulation of microtubule dynamics is essential for many vital cellular processes such as morphogenesis and motility and Cdk5-p35 complex co-exists with

microtubules in the brain (Sobue *et al*., 2000; Paudel *et al*., 1993). Since we have identified CK2 as a p35-interacting protein and previous studies have implied that CK2, a ubiquitously expressed protein kinase involved in diverse cellular functions (Litchfield, 2003; Meggio and Pinna, 2003), may be involved in regulating cytoskeleton reorganization (Serrano *et al*., 1987; Diaz-Nido *et al*., 1988; Serrano *et al*., 1989), we therefore investigated if CK2 is also involved in mediating microtubule dynamics. The CK2 holoenzyme is composed of two catalytic α or α' subunits and two regulatory β subunits. We showed that the α subunit of CK2 binds directly to both microtubules and tubulin heterodimers. The CK2 holoenzyme (but not its individual subunits) exhibited a potent effect in inducing microtubule assembly and bundling. Moreover, polymerized microtubules were strongly stabilized by CK2 against cold-induced depolymerization. In addition, the kinase activity of CK2 is not required for its microtubule-assembly and stabilizing function since a kinase-inactive mutant of CK2 displayed similar microtubule-assembly activity as the wild-type. Knockdown of CK2α/α' in cultured cells by RNA interference dramatically destabilized their microtubule networks. The destabilized microtubules were thus readily disrupted by colchicine at a very low concentration. Further, over-expression of chicken CK2α or its kinase-inactive mutant in CK2α/α'-depleted cells fully restored microtubule resistance to low doses of colchicine. To our knowledge, these findings demonstrate for the first time that CK2 is a microtubule-associated protein that confers microtubule stability in a phosphorylation-independent manner.

Section 1

Introduction

1. Introduction

The ability of cells to function and proliferate depends largely on their response to the immediate intracellular environments as well as the external stimuli. These cellular signals trigger a specific set of mechanisms within the cells to bring about a change in cell function. These mechanisms are highly regulated to control cellular functions, commonly by means of changes in protein conformation. A process of signal transduction conveys the external message to the internal cellular organelles.

Protein phosphorylation is one such mechanism, and is catalyzed by enzymes known as protein kinases, while protein phosphatases catalyze the reverse process, dephosphorylation (Cohen, 2002; Hunter, 2000). Protein kinases are classified into the serine/threonine-specific, tyrosine-specific, histidine-specific or the dual specificity (Ser/Thr and Tyr) class of kinases, depending on the residue being targeted for phosphorylation. All known protein kinases of this class share a related catalytic domain and are distinguished by their unique regulatory domains.

Mammalian brains are highly compartmentalized into groups of functionally specialized neurons. Cell migration and neurite outgrowth must be tightly orchestrated to achieve this level of organization. Likewise, cellular processes such as DNA replication and cell division must also be tightly regulated during embryogenesis to produce a viable organism. Many protein kinases have emerged to be important regulators of these processes. Together, they play distinct roles in coordinating the transition of different cellular functions.

1.1 Protein Kinase CK2: Composition and Structure

Casein kinases are multifunctional, highly conserved, serine/threonine protein phosphotransferase that are ubiquitous in yeast and higher eukaryotes (Pinna, 1990). They are cyclic-nucleotide-independent protein kinases that preferentially phosphorylate acidic proteins (Hathaway and Traugh, 1982). Two distinct casein kinases have been found in many different cell types. They have been designated casein kinase 1 (CK1) and casein kinase 2 (CK2) according to the elution profile obtained by diethylamino-ethylcellulose (DEAE-cellulose) chromatography (Hathaway and Traugh, 1979).

Protein kinase CK2 is an oligomeric enzyme with molecular mass (M_r) of 130-150 kDa, as determined by sedimentation velocity and equilibrium analysis (Hathaway and Traugh, 1979; Pinna, 1990), with the exception of a porcine liver CK2 of 210 kDa (Baydoun *et al*., 1986) and a monomeric human spleen CK2 of 43 kDa (Gounaris *et al*., 1987). The holoenzyme of CK2 consists of subunits α, α' and β which associate to form several distinct heterotetramers, namely α2β2, α'2β2 and αα'β2. A number of studies have reported that these subunits may also exist individually in the cells which are devoid of their counterparts (Stigare *et al*., 1993; Heriche *et al*., 1997; Kusk *et al*., 1999). The reported *M*r for the β subunit, as determined by gel electrophoresis in sodium dodecyl sulfate (SDS), is usually 24-26 kDa, while the values for the α and α' subunits ranges from 35 to 44 kDa (Hathaway and Traugh, 1982; Litchfield *et al*., 1990).

The α, α' and β subunits are the products of three distinct genes (Allende and Allende, 1995). All subunits have an extraordinarily high degree of evolutionary conservation. Firstly, the sequence of the α subunit is largely conserved across mammalians and other species (*Drosophila*, chicken, mouse, rat, pig, bovine, human) where sequence identity ranges from 67 to 90%. Secondly, the α and α' subunits are structurally very homologous, despite the differences in their C-terminal regions (Lozeman *et al*., 1990). Thirdly, the cDNA sequences of the β subunit of *Drosophila*, mouse, rat, pig, bovine and human are also highly homologous (Pinna, 1990).

The α and α' subunits are catalytically active, whereas the β subunit is inactive. Identification of catalytic subunits CK2α and CK2α' was based on their enzymatic activity in the absence of the β subunit. The functions of the β subunit are to confer stability, regulate the enzymatic activity of the holoenzyme and the specificity of the catalytic subunit (Faust and Montenarh, 2000). The catalytic domain of the α subunit is homologous to the catalytic domains of other protein kinases. There is a short N-terminal segment, termed "glycine-loop on phosphate anchor", which makes contact with the β-phosphate of the bound ATP and is involved in the recognition of peptide substrates (Fig. 1). A stretch of basic residues, which is probably the most striking hallmark of CK2, is located just downstream from the invariant lysine (Lys-68) and is recognized as an essential residue involved in ATP binding in all protein residues. This high concentration of adjacent basic residues is unique among protein kinases in this region. This is the region which interacts with the β subunit and is involved in the down-regulation by the β subunit towards some substrates (Marin *et al*., 1997; Sarno *et al*., 1998). In addition, it is also implicated in the inhibition of CK2 by heparin (Vaglio *et al*., 1996). Another intriguing function for

this basic sequence is based on the fact that it falls into the description of a nuclear localization signal (NLS) that are known to mediate the attachment of molecules to transporter proteins for their regulated nuclear import (Boulikas, 1996). It is interesting to note that none of the other protein kinases possess a NLS motif with more than three basic residues. Although it cannot be fully excluded that the NLS sequences of α and α' subunits have little to do with nuclear transport since these subunits lack an acidic stretch close to the NLS which enhances binding with transporter proteins (Boulikas, 1996), it is nevertheless possible that these strong NLS-like motifs allow targeting of the kinase to the nucleus ascribed to this kinase (Rihs *et al*., 1991). The next segment contains the functional elements termed the 'activation loop', followed by a C-terminal tail that has been shown to be phosphorylated by the protein kinase $p34^{cdc2}$ (Litchfield *et al*., 1992).

Within the N-terminal of the β subunit of CK2 lies an autophosphorylation site, which has been shown to be phosphorylated readily *in vitro* (Meggio *et al*., 1989). A cyclin-like 'destruction box' also lies within this region of the protein, which is followed by an acidic region known to be responsible for the intrinsic down-regulation of CK2 (Meggio *et al*., 1994). The C-terminal segment is responsible for β-β dimerization, association with the α subunit, protection against denaturation and proteolysis, and up-regulation of activity (Boldyreff *et al*., 1993; Boldyreff *et al*., 1996; Marin *et al*., 1997; Krehan *et al*., 1996). In addition, this region possess a phosphorylatable Ser209 which has been shown to be phosphorylated by $p34^{cdc2}$, although no physiological role has been implicated for this modification (Litchfield *et al*., 1992).

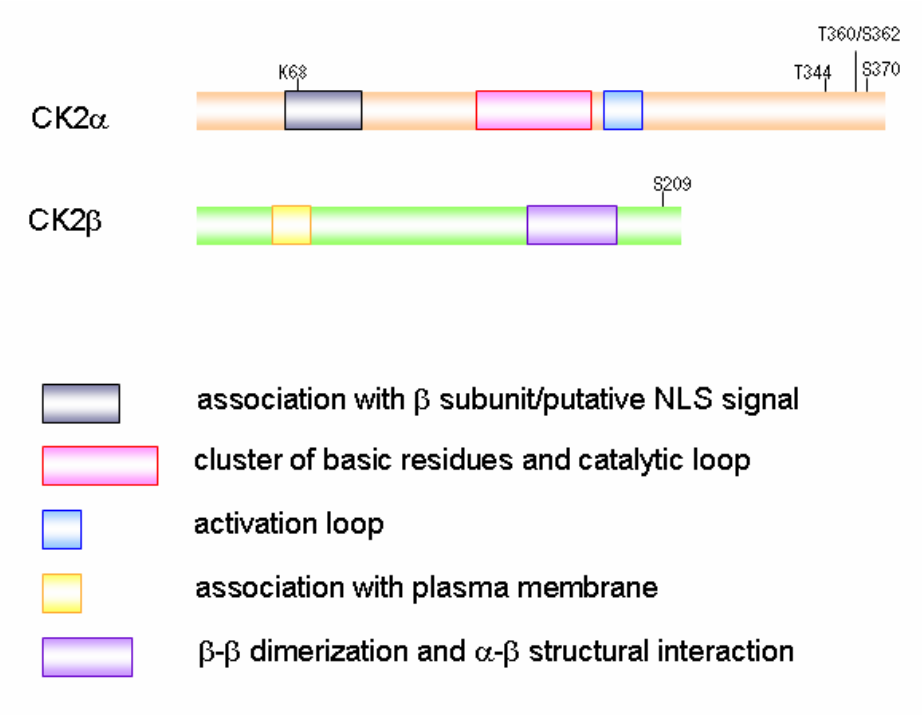

FIG. 1. Schematic diagram depicting various motifs of the CK2 proteins. Within the α subunit of human CK2, the domain in grey includes the invariant Lys-68 which is involved in ATP binding, followed by a cluster of basic residues which associates with the β subunit, and a strong karyophilic putative NLS signal. The domain in pink comprises a series of six basic residues regularly spaced, which appear to be involved in the recognition of peptide substrates, and the catalytic loop. The domain in blue contains the functional elements termed 'activation loop'. There are four phosphorylatable residues (Thr-344, Thr-360, Ser-362 and Ser-370) which are identified as potential targets for Cdc2 (Litchfield *et al*., 1992). Within the β subunit of human CK2, the domain in yellow is highly conserved between organism, and it includes seven acidic residues and is responsible for negative regulation of CK2 and its association with plasma membrane. The domain in purple is mainly responsible for β-β dimerization and α-β structural interaction. There is a phosphorylatable serine residue (Ser-209) at the C-terminal end (Litchfield *et al*., 1992).

1.1.1 Tissue-specific distribution and subcellular localization

CK2 is expressed by various species at different stages of development. In fact, almost all tissues of all higher organisms express CK2. By *in situ* hybridization and CK2 transcripts, Mestres and co-workers showed that both CK2 protein subunits were detected in nearly all organs of the mouse embryo, suggesting a general role during embryonic development (Mestres *et al*., 1994). In general, the level of CK2 transcripts correlates with protein expression. A survey was also made of the activity of CK2 in extracts made from various tissues of adult rat. The highest activity was found in brain, testis and liver, whereas CK2 activity in kidney and spleen is low (Singh and Huang, 1985; Nakajo *et al*., 1986; Guerra *et al*., 1999). CK2 activity, as well as its immunoreactivity, were also present in all brain regions studied (Girault *et al*., 1990). CK2 activity was also studied in mouse cortex and caudate-putamen during development (De Camilli and Greengard, 1986). Its levels was found to be high at embryonic day 16 and during the early post-natal period, and appeared to decrease slightly in the adult.

Numerous workers have investigated the subcellular localization of CK2 though many of the initial results were somewhat confusing. However, it turned out that CK2 was present not only in the nucleus and the cytoplasm (as reported initially) but nearly everywhere in the cell. Oligomeric forms of CK2 were observed to be closely associated with the plasma membranes prepared from A431 cells and from SF9 insect cells expressing the catalytic and regulatory subunits of CK2 (Sarrouilhe *et al*., 1998). The holoenzyme seems to be targeted to the plasma membrane by the β-subunit of CK2. On the other hand, others have reported that CK2 activity and its immunoreactivity as predominantly cytoplasmic (Singh and Huang, 1985; Girault *et*

al., 1990). Using immunofluorescence and immunoelectron microscopy, Yu and coworkers showed that CK2α and CK2β are localized to the cytoplasm during interphase and are distributed throughout the cell during mitosis (Yu *et al*., 1991). In contrast, CK2α' is localized in the nucleus during G1 phase and in the cytoplasm during the S phase (Yu *et al*., 1991). Likewise, CK2 immunoreactivity had been reported to be either associated with the nucleus or distributed between the nucleus and cytoplasm (Schneider and Issinger, 1989). It is apparent that the variety of nuclear substrates found for CK2 makes a nuclear/nucleolar function for this protein kinase likely (Meggio and Pinna, 2003).

A closer look at the subcytosolic localization of CK2 reveals that this enzyme is also associated with cytosolic organelles. CK2 has been purified from bovine kidney mitochondria (Damuni and Reed, 1988). An *in vivo* localization of CK2 at the membrane of the mitochondria seems to be reasonable since some potential substrates of CK2 are localized in the matrix of mitochondria (Meggio and Pinna, 2003). CK2 has been identified as an endoplasmic reticulum (ER)-associated kinase responsible for the *in vitro* phosphorylation of calnexin and signal sequence receptor-α (Ou *et al*., 1992). These proteins are implicated to function as chaperones in the ER. Moreover, the cytosolic domain of calnexin was found to be phosphorylated *in vivo* at CK2 sites Ser534 and Ser 544 and these modifications play a role in targeting calnexin to the ribosomes (Chevet *et al*., 1999).

1.1.2 Regulation of CK2

CK2 was initially isolated as a cyclic nucleotide-independent protein kinase that preferentially phosphorylates acidic proteins (Hathaway and Traugh, 1982), which led to much debate and controversy over its regulation in cells (Litchfield *et al*., 1994). The fact that CK2 activity is generally detected in cell or tissue extract even in the absence of any stimulation or addition of cofactors, or when it is expressed in bacteria, lends itself to the conclusion that CK2 is constitutively active or unregulated. Till now, studies reporting on the activation of CK2 in response to a diverse array of stimuli have not yielded any consistent insights into the mechanisms responsible for CK2 regulation in cells. Some of the mechanisms that contribute to the regulation of CK2 in cells include regulated expression and assembly, modulation by covalent modification and regulatory interactions with protein and/or non-protein molecules.

In the case of the Cdks, it is evident that their kinase activity is absolutely dependent on the presence of regulatory cyclin subunit (Pines, 1995). In this respect, CK2β is analogous to the cyclins that it modulates the catalytic activity and substrate specificity of CK2 as well as the assembly of CK2 complexes. The existence of a putative destruction box within the sequence of CK2β and the demonstration that CK2β is ubiquitinated and degraded through a proteasomal pathway further emphasizes its potential similarities with the cyclins (Zhang *et al*., 2002a). Furthermore, it has been reported that CK2 activity oscillates during the cell cycle, analogous to the Cdks (Carroll and Marshak, 1989; Bosc *et al*., 1999). Generally, it appears that CK2 levels correlate to proliferation rate, as cells with higher proliferation rates generally exhibit higher levels of CK2 (Munstermann *et al*., 1990).

As noted above, CK2 has traditionally been considered a tetrameric enzyme, with CK2β exerting control over the catalytic activity of CK2 at a number of possible levels. However, there is mounting evidence to suggest that the catalytic subunits of CK2 exist outside the tetrameric CK2 holoenzyme. It is intriguing that there are substrates which can be phosphorylated by CK2α or by CK2α' but not the tetrameric CK2 (Marin *et al*., 1999; Litchfield, 2003). There is a possibility that tetrameric CK2 complexes undergo regulated disassembly in cells. This is supported by recent studies on the dynamic localization of individual CK2 subunits showing independent movements of CK2α and CK2β within cells (Martel *et al*., 2001; Filhol *et al*., 2003). Furthermore, recent crystal structure of tetrameric CK2 revealed that the surface contact between the catalytic and regulatory subunits were considerably fewer than those typically observed in stable protein complexes (Niefind *et al*., 2001). In this respect, CK2 may indeed undergo regulated disassembly and reassembly in cells (Allende and Allende, 1998).

For many protein kinases, it is apparent that stimulus-dependent phosphorylation of sites within an activation loop is required for their activation. By comparison, the catalytic subunit of CK2 exhibit robust activity when expressed in bacteria in either presence or absence of CK2β (Grankowski *et al*., 1991; Hinrichs *et al*., 1993). Similarly, there has been limited support for the suggestion that phosphorylation regulates the activity of CK2 in response to cellular stimulation (Agostinis *et al*., 1987; Ackerman *et al*., 1990; Mulner-Lorillon *et al*., 1990; Palen and Traugh, 1991; Litchfield *et al*., 1991). Taken together, these data indicate that phosphorylation is not absolutely required to activate CK2. On the other hand, both CK2α and CK2β are phosphorylated in a cell cycle-dependent manner (Litchfield *et*

al., 1992; Litchfield *et al*., 1991). Though these sites do not appear to directly effect a dramatic change in the catalytic activity of CK2, they may, by controlling the stability of CK2β autophosphorylation, indirectly regulate cellular CK2 activity (Zhang *et al*., 2002a). The C-terminal phosphorylation of CK2α may also regulate CK2 indirectly through interaction of phosphorylated CK2α with the peptidyl-prolyl isomerase Pin1 (Messenger *et al*., 2002). Interactions between Pin1 and CK2 do not appear generally to influence CK2 activity, but do inhibit the CK2-catalyzed phosphorylation of topoisomerase IIα *in vitro*.

CK2 is typically known to be independent of those small molecules that are involved in the activation of second messenger-dependent kinases. However, it has been established that CK2 is inhibited by negatively-charged compounds such as heparin and activated by positively-charged compounds such as polyamines (Tuazon and Traugh, 1991). Further finding that CK2 level and activity were elevated in mice with enhanced polyamine levels, resulting from forced overexpression of ornithine decarboxylase, supports the possibility that CK2 levels can indeed be modulated by polyamines *in vivo* (Leroy *et al*., 1997).

A large body of evidence indicates that protein-protein interactions represent a major mechanism for the regulation of specific protein kinases (Pawson and Nash, 2000). The identification of several proteins that interact with CK2 is consistent with this conjecture that CK2 may be directly, or indirectly, regulated by interacting proteins. CK2 interacts with proteins such as fibroblast growth factor 1 and HSP-90 that may directly alter or stabilize its catalytic activity (Skjerpen *et al*., 2002; Miyata and Yahara, 1995). Studies have demonstrated that CK2 also interacts with other

proteins, such as tubulin and *FAS*-associated factor 1, that may be involved in the targeting of CK2 to specific sites or structures within the cells (Faust *et al*., 1999; Jensen *et al*., 2001). Overall, it is evident that many distinct mechanisms may contribute to the regulation of CK2 in the cells. In this respect, it is conceivable that many distinct, independently regulated subpopulation of CK2 exist in cells in order to carry out its myriad of cellular functions.

1.1.3 Biological effects of CK2

In the last few decades, a great deal of research has been devoted in the study of CK2 and its cellular implications (Litchfield, 2003; Meggio and Pinna, 2003). By its interaction with more than 300 binding partners and substrates, CK2 modulates the action of proteins that are involved in cell signaling and adhesion, cytoskeletal structure, synaptic-vesicle recycling, as well as transcriptional machineries. Moreover, CK2 is instrumental and necessary for promoting cell survival (Litchfield, 2003; Ahmed *et al*., 2002), which further substantiates the mandatory roles of CK2 in the cells.

1.1.3.1 Regulation of adhesive proteins

Studies have shown that phosphorylation might function as a regulatory mechanism for adhesive components of the cell (Stepanova *et al*., 2002; Serres *et al*., 2000; Seger *et al*., 1998). Till now, the phosphorylation by CK2 has been linked to the functions of several cell adhesion molecules, including vitronectin and E-cadherin.

Vitronectin, a secretory product of the astrocytes, is known to be an important adhesive glycoprotein. It participates in the regulation of the complement function and promotes cell attachment spreading and migration through an Arg-Gly-Asp (RGD) sequence that is known to be recognized by integrins, one type of the adhesive transmembrane receptors present in focal adhesions (Hynes, 2002). Interestingly, vitronectin was unearthed to be a substrate of CK2. The phosphorylation by CK2 on vitronectin is selectively targeted to two threonine residues that are vicinal to the RGD sequence, resulting in a significant modulation of cell adhesion (Seger *et al*., 1998). Hence, CK2 phosphorylation converts vitronectin from cellular ‘glue’ to a

cellular ‘super glue’. More recently, it was discovered that this phosphorylation increases cell adhesion via the $\alpha_v\beta_3$ integrin and this event is phosphatidylinositol 3-kinase-dependent (Seger *et al*., 2001).

Among the many types of cell-cell adhesion molecules, cadherins play a critical role in establishing adherens-type junctions by mediating Ca^{2+}-dependent cell-cell adhesion (Takeichi, 1995; Huber *et al*., 1996; Yagi and Takeichi, 2000). Cadherin-based cell-cell adhesion is critically involved in early embryonic morphogenesis, as exemplified by the early embryonic lethality of mice lacking E-cadherin, a prototype classical cadherin (Riethmacher *et al*., 1995; Larue *et al*., 1994). β-catenin, a member of the Armadillo repeat protein family, binds directly to the cytoplasmic domain of E-cadherin, linking it via α-catenin to the actin cytoskeleton. E-cadherin has been shown to be a substrate of CK2 and phosphorylation of the E-cadherin cytoplasmic domain by CK2 appears to modulate the affinity between β-catenin and E-cadherin, ultimately modifying the strength of cell-cell adhesion (Serres *et al*., 2000; Lickert *et al*., 2000).

1.1.3.2 Regulation of cytoskeletal elements

CK2 can phosphorylate a variety of cytoskeletal proteins, including β tubulin, myosin heavy chain, spectrin, tau, MAP-1A and MAP-1B (Meggio and Pinna, 2003), but the role of these post-translational changes is still not well understood. In neuronal cells, one of the proposed roles for these changes has been the promotion of neuritogenesis, which involves elongation and maturation of both axonal and dendrite arbors. Depletion of CK2 with anti-sense oligonucleotide causes a site-specific dephosphorylation of MAP-1B and blocks neuritogenesis in neuroblastoma cells

(Ulloa *et al*., 1993). In line with these findings, a CK2-related activity was also found to phosphorylate brain MAP-1B *in vitro*. More importantly, brain MAP-1B phosphorylated *in vitro* by CK2 was shown to coassemble efficiently with microtubule proteins in the same way as *in vivo* phosphorylated MAP-1B from neuroblastoma cells (Diaz-Nido *et al*., 1988). These results led to the proposal that MAP-1B phosphorylation by CK2 may favor microtubule nucleation and stabilization during neurite outgrowth. In addition, CK2 phosphorylates a neural-specific isoform of tubulin, preferentially in the polymerized form (Serrano *et al*., 1987; Diaz-Nido *et al*., 1990), though no physiological relevance has been shown so far. However, it has been suggested that CK2-induced MAP-1B phosphorylation is a prior step to CK2-induced tubulin phosphorylation.

1.1.3.3 Regulation of substrates involved in signal transduction

Many substrates of CK2 are involved in signal transduction pathways (Meggio and Pinna, 2003). $p34^{cdc2}$ is phosphorylated by CK2 during G1 phase of the mammalian cell cycle (Russo *et al*., 1992). In addition, the α and β subunits of mammalian CK2 are phosphorylated *in vitro* by $p34^{cdc2}$ and their phosphorylation increases dramatically in cells arrested at mitosis (Litchfield *et al*., 1992; Litchfield *et al*., 1991). Unfortunately, nothing has been reported about the effect of these modifications. p53 is one of the most powerful negative regulators of growth. CK2 phosphorylates murin p53 at Ser386, which has been shown to control several independent functions of p53 including site-specific DNA binding, strand renaturation, transcription repression and anti-proliferative effect (Agarwal *et al*., 1998). Recently, studies have reported that Ser386 is highly resistant to dephosphorylation, suggesting that, once phosphorylated at this CK2 site, p53 remains in an activated form throughout its lifespan (McKendrick *et al*., 1999).

Synergistic phosphorylation by glycogen synthase kinase-3 (GSK-3) and CK2 has been demonstrated in glycogen synthase, regulatory subunit (R2) of PKA and the inhibitor-2 of PP1 (DePaoli-Roach *et al*., 1981; DePaoli-Roach, 1984; Meggio *et al*., 1981; Carmichael *et al*., 1982; Hemmings *et al*., 1982; Edelman *et al*., 1987). Evidence is that prior serine/threonine phosphorylation of glycogen synthase, inhibitor-2 and R2 by CK2 makes these proteins better substrates for GSK-3. It appears that free CK2α, by phosphorylating protein phosphatase 2A (PP2A) and thence by stimulating its activity, could indirectly cause down-regulation of the PP2A substrate MEK and thus block the activation of the Raf-MEK-MAPK kinase cascade (Heriche *et al*., 1997). Raf activation-dependent disruption of the CK2α-PP2A complex might be indeed a necessary step for maximal activation of the MAP kinase pathway (Lebrin *et al*., 1999). Likewise, CK2 phosphorylation of stathmin, a microtubule depolymerizing factor, remains unclear functionally. Recent work has shown that the microtubule depolymerization activity of unphosphorylated stathmin is slightly enhanced if the protein is phosphorylated by CK2 prior to depolymerization assays *in vitro* (Moreno and Avila, 1998). However, no apparent *in vivo* CK2 phosphorylation of stathmin has been detected.

1.1.3.4 Regulation of proteins associated with synaptic vesicle recycling

A number of phosphoproteins are associated with synaptic vesicles and appear to be involved in neurotransmitter release (Sudhof, 1995). Among these components, synaptotagmin, syntaxin and dynamin I can be phosphorylated by CK2 (Bennett *et al*., 1993; Robinson *et al*., 1994). Synaptotagmin is a single transmembrane protein that contains a cytoplasmic phospholipid-binding region. This region is involved in

mediating the interaction of synaptic vesicles with the presynaptic plasma membrane. Syntaxin, as a neuronal protein at the synaptic sites, appears to mediate the interaction of synaptotagmin with the N-type calcium channel, possibly providing a mechanism for docking synaptic vesicles at the presynaptic membrane (Littleton and Bellen, 1995). Dynamin I has received prominent attention as a result of its protein kinase C (PKC) phosphorylation on repolarization-induced calcium removal and its dephosphorylation on depolarization-induced calcium influx accompanying synaptic vesicle recycling. Interestingly, CK2 phosphorylates the phospholipid-recognizing site of synaptotagmin. Furthermore, CK2 phosphorylation of dynamin I prevents phosphorylation by PKC, providing a model for potential interaction between distinct signaling pathways in the presynaptic regulation of endocytosis and exocytosis of synaptic vesicles (Robinson *et al*., 1994). It is therefore conceivable that CK2 phosphorylation of synaptotagmin, syntaxin and dynamin I may represent one mechanistic basis of increasing presynaptic efficacy through the regulation of the synaptic vesicle-membrane trafficking.

1.1.3.5 Regulation of transcription factors

A cursory examination of CK2 substrates (Meggio and Pinna, 2003) reveals that a number of them are involved in regulation of genes, including *c*-Jun, *c*-Fos, *c*-Myc, Max, Sp1 and p53. Phosphorylation by CK2 has been found to affect either positively or negatively binding of these sequence-specific transcription factors. The early signal transducer *c*-Jun is a major component of the inducible complex AP1 that binds to tumor-promoting agent response element either as a homodimer or as a heterodimer with other Jun or Fos proteins (Hunter and Karin, 1992). *c*-Jun is phosphorylated on six major sites (Hunter and Karin, 1992; Lin *et al*., 1992), and so far, only phosphorylation by CK2 and GSK-3 has been shown to inhibit *c*-Jun-DNA

binding. *In vitro*, CK2 phosphorylates Thr231 and Ser249, which are the negative regulatory C-terminal sites located immediately to the N-terminal side of its DNA-binding domain (Hunter and Karin, 1992; Lin *et al*., 1992).

Max is a DNA-binding protein that can form homodimer and heterodimer with members of the Myc proto-oncogene family. The DNA-binding capability of Max/Myc heterodimer binding is unaffected (Berberich and Cole, 1992). It is therefore possible that CK2 phosphorylation of Max may have an important role in modulating or eliminating any potential competition for Max/Myc target genes by the Max homodimers.

1.2 Cyclin-Dependent Protein Kinase Family

The regulation of cell cycle progression is a tightly controlled process. The timing through the various phases of G1/S/G2 and M is mediated through an ordered progression of Cdk activation (Morgan, 1997; Pines, 1999). Cyclin-dependent kinases (Cdks) are the cell cycle-associated protein kinases that regulate proliferation, differentiation, senescence and apoptosis (Li and Blow, 2001). Members of the Cdk family are proline-directed serine/threonine kinases (30-35 kDa) whose activities are controlled through a complex series of regulatory mechanisms, including binding to their appropriate cyclin partners, activating and inactivating phosphorylation modifications, and endogenous inhibitors of the Cdk activity (Morgan, 1997; Tannoch *et al*., 2000). In general, each Cdk periodically interacts with a specific subset of cyclins to regulate the Cdk activity. There are at least nine different Cdks (Cdk1-Cdk9) and many more cyclins (cyclin A through T). Cyclin/Cdk complexes are in turn regulated in defined stoichiometric combinations with specific small inhibitory proteins, the Cdk inhibitors (CKIs). There are two families of CKIs: the INK4 (inhibitor of Cdk4) family members, $p16^{ink4a}$, $p15^{ink4b}$, $p18^{ink4c}$ and $p19^{ink4d}$, specifically inhibit cyclin D-associated kinases, and the KIP (kinase inhibitor protein) family members, $p21^{cip1/waf1}$, $p27^{kip1}$ and $p57^{kip2}$, bind and inhibit the activity of cyclin E/Cdk2, cyclin A/Cdk2 and cyclin B/Cdk1 complexes (Sherr and Roberts, 1999; Pines, 1995).

Generally, members of Cdk family share greater than 40% sequence identity and have a cyclin-binding and -activating domain (Morgan, 1995). The typical Cdk subunit contains a 300 amino acid catalytic core that is completely inactive when

monomeric and unphosphorylated. Cellular Cdks levels tend to remain in constant excess throughout the normal cell cycle, and regulation of catalytic activity is primarily post-translational.

Kinase activity of all Cdks requires the binding of a positive regulatory cyclin subunit. Homology among cyclins is often limited to a region of about 100 amino acids, which adopts a conserved structural domain called the cyclin-box fold, and this region is required for Cdk binding and activation. Each phase of the cell cycle is characterized by the expression of distinct type of cyclins, and fluctuations in cyclin levels represent the primary mechanism by which Cdk activity is regulated. Cyclins are thought to contain regions that target the Cdk to specific substrates or subcellular localizations. In addition to simply activating the associated Cdk, they can thus promote activity towards specific substrates (Hoffmann *et al*., 1993; Peeper *et al*., 1993; Dynlacht *et al*., 1994). The enhanced activity of certain complexes is probably due to positive interactions between the cyclin and the substrate.

The assembly of a Cdk with its corresponding cyclin yields only a partially active complex, full activity being achieved only after phosphorylation of the Cdk on a conserved threonine residue proximal to the ATP-binding cleft (Kaldis, 1999). Cyclin-Cdk binding precedes the activating phosphorylation on the threonine residue located at the T-loop, a region of amino-acids that blocks access of ATP to the catalytic domain. The crystal structure of the human Cdk2 apoenzyme shows that it is held in an inactive state by at least two major structural restraints (De Bondt *et al*., 1993; Morgan and De Bondt, 1994). Firstly, the substrate binding site is blocked by an extended loop termed the T loop. Secondly, side chains in the ATP-binding site are

oriented so that the ATP phosphates are poorly positioned for efficient phosphate group transfer. Analysis of the crystal structure of cyclin A/Cdk2 complexes indicates the cyclin/Cdk interaction causes a conformational change in the Cdk, making the T-loop more accessible for the activation phosphorylation (Russo *et al*., 1996; Jeffrey *et al*., 1995). The phosphorylation causes a further conformational change in the T-loop, making the catalytic cleft fully accessible to ATP. In addition to stimulating kinase activity, the activating threonine phosphorylation has also been suggested to enhance the stability of some cyclin/Cdk complexes (Ducommun *et al*., 1991; Desai *et al*., 1995).

In mammals, Cdk-cyclin complexes can be inhibited by phosphorylation at two sites near the amino terminus. Phosphorylation of Cdk1 (Cdc2) and Cdk2 at Thr14 and Tyr15 by the dual-specificity kinases Wee1, Myt1 and Mik1 inhibits their activities (Mueller *et al*., 1995b; Mueller *et al*., 1995a; Watanabe *et al*., 1995), and this is particularly important in the control of Cdc2 activation during mitosis. Thr14 and Tyr15 are both dephosphorylated by a dual-specificity phosphatase termed Cdc25 whose activity is enhanced during mitosis. Another mechanism for Cdk regulation involves a diverse family of proteins termed the CKIs. Most CKIs bind tightly to Thr160/161-phosphorylated Cdk-cyclin complexes (Thr160 of Cdk2 and Thr161 of Cdk1) and directly inhibit kinase activity.

Though most known Cdks are involved in cell cycle control, this definition of Cdks does not limit their biological function (Morgan, 1995). The classical Cdks are also involved in regulating numerous neuronal processes such as differentiation, senescence, and apoptosis through modification of gene transcription (Tannoch *et al*.,

2000). In proliferating cells, aberrations in the regulation of Cdks is associated with tumor formation, whereas their disappearance/inhibition in precursor of neuronal cells coincides with terminal differentiation (Okano *et al*., 1993).

1.3 Neuronal Cdk5 kinase

Since its discovery in the early 1990s, Cdk5 has emerged to be an important regulator of neuronal migration in the developing central nervous system (CNS) (Dhavan and Tsai, 2001). Cdk5 was initially identified independently by virtue of its close sequence homology to human Cdk1, by biochemical purification from bovine brain based on its proline-directed Ser/Thr kinase activity and by affinity isolation as a cyclin D1-associated protein in fibroblasts (Meyerson *et al*., 1992; Lew *et al*., 1992a; Xiong *et al*., 1992). Cdk5 is an atypical member of the Cdk family. Despite its close sequence homology with Cdk1, it is not activated by any known cyclins, although it can bind cyclin D1 and cyclin E (Xiong *et al*., 1992; Miyajima *et al*., 1995) . The first known activators of Cdk5 are p35 (Fig. 2) and its proteolytic product p25, which were isolated as a binding partner of Cdk5 in the brain extract (Lew *et al*., 1994). p25 is a 208 residues peptide derived from carboxyl terminal of p35 and it retains the Cdk5-binding and activating domain of p35 (Zheng *et al*., 1998; Qi *et al*., 1995). Another activator of Cdk5, p39, was identified by its sequence homology to p35, with which it shares 57% amino acid identity (Tang *et al*., 1995; Humbert *et al*., 2000a). Monomeric Cdk5 does not display any enzymatic activity. The binding to p35, p25 or p39 activates its kinase activity in the absence of any Cdk5 modification and association of any other protein factors (Lew *et al*., 1994; Tsai *et al*., 1994; Ishiguro *et al*., 1994). Though Cdk5 is a ubiquitously expressed protein, its kinase activity is restricted to the nervous system by the neuron-specific expression of its activators p35 and p39 (Ko *et al*., 2001). Interestingly, several groups have since demonstrated the presence of active Cdk5 in many non-neuronal tissues (Dhavan and Tsai, 2001).

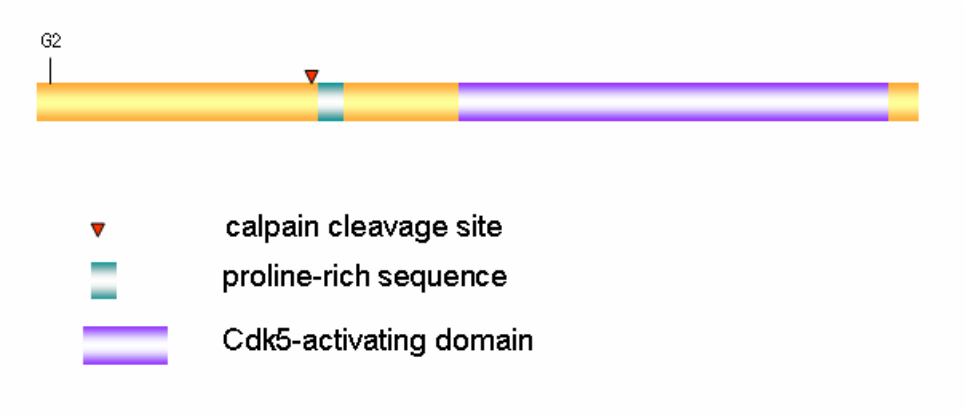

FIG. 2. Schematic diagram depicting motifs of p35. Within the human p35 sequence, there is an N-terminal myristoylation site (Gly-2). This is followed by a calpain cleavage site, which cleaves p35 into p25 as well as an N-terminus polypeptide (p10). A short proline-rich sequence, with no reported function, follows the calpain cleavage site. The C-terminus of p35 constitutes the Cdk5-binding and activation domain, which is present in both p35 and p25.

Although Cdk5 is a member of the Cdk family, it is not involved in cell cycle regulation. Since its discovery more than a decade ago, Cdk5 has been shown to play an important role in many cellular processes occurring within neurons in the CNS (Homayouni and Curran, 2000). For example, Cdk5 is known to participate in the regulation of cytoskeleton organization, axon guidance, membrane transport, synaptic function, dopamine signaling and drug addiction (Dhavan and Tsai, 2001). Gene targeting experiments have demonstrated an essential role for Cdk5 in the cellular organization of the CNS. Mice that are deficient of *Cdk5* die just before or after birth, and show widespread disruptions in the neuronal layering of many brain structures (Ohshima *et al*., 1996; Gilmore *et al*., 1998; Ohshima *et al*., 1999). The lethality of the *Cdk5*-deficient mice is likely to be a result of defects in the nervous system, since it can be completely rescued by expressing the *Cdk5* transgene under the p35 promoter (Tanaka *et al*., 2001). Contrary to *Cdk5*-deficient mice, $p35^{-/-}$ mice are viable and fertile, though they have increased susceptibility to seizures (Chae *et al*., 1997; Kwon and Tsai, 1998). *p35*-deficient mice show an inverted layering of cortical neurons comparable to that observed in the $Cdk5^{-/-}$ mice, but have only mild

disruptions in the hippocampus and have a fairly normal cerebellum. *p39/p35* double knockout display the same phenotype as *Cdk5*$^{-/-}$ mice, further establishing these proteins as the primary activators of Cdk5 (Ko *et al*., 2001). The viability of the *p35/p39*$^{-/-}$ mice may dictate that p35/p39 may perform other cellular functions that are independent of its activation of Cdk5.

Cdk5, p35 and p39 are abundantly expressed in adult brains and high levels of Cdk5 kinase activity are detected in post-mitotic neurons of the nervous system in accordance with the expression pattern of p35 and p39. As early as E10, a restricted expression of p35 in developing CNS of mouse embryos had been observed by *in-situ* hybridization. Expression studies of E12 and E15 mouse brains revealed that there is no p35 in proliferating neuronal precursors but that it is expressed in post-mitotic neurons of the developing cortex (Delalle *et al*., 1997; Ino *et al*., 1994; Tsai *et al*., 1993). As neurons differentiate, cell cycle Cdks are down-regulated while the Cdk5 activity is increased (Zheng *et al*., 1998). p35 is highly expressed in the post-mitotic neurons of developing cortex but is not found in proliferating neuronal precursors. On the other hand, the highest level of p39 expression in the CNS occurs postnatally. Apparently, p35 and p39 display an overlapping, but distinct temporal and spatial pattern of their expression in the brain (Delalle *et al*., 1997). Thus, Cdk5-p39 may arbitrate functions distinct from those involving Cdk5-p35 during neurodevelopment.

Cdk5 plays an indispensable role in the central nervous system. Recently, the crystal structure of Cdk5-p25 has been reported (Tarricone *et al*., 2001). p25 binds Cdk5 in the region of the PSSALRE helix in the kinase small lobe and there are also extensive contacts between p25 and the activation loop (T-loop) of the kinase. Despite

its partial structural similarity with the cyclins, p25 displays an unprecedented mechanism for the regulation of a cyclin-dependent kinase. p25 tethers the unphosphorylated T-loop of Cdk5 in the active conformation. Biochemical data showed that Ile 153 and Ser 159 (equivalent to Thr 160 in Cdk2) in the T-loop of Cdk5 are critical for p35 interaction. Substitution of Ser159 to a threonine residue prevents p35 binding, while a substitution to an alanine residue affects neither binding nor kinase activity. Cdk5 is a proline-directed kinase where it has a strong preference for positively charged residues in the +3 position. For Cdk2-cyclin A, the phosphorylation of Thr160 is essential to encode this specificity. Biochemical and structural studies showed that Glu240 in p35 plays an inevitable role in the recognition of the basic residue in the +3 position, indicating that p35 directly participates in substrate recognition (Tarricone *et al*., 2001).

It has been shown that cellular Cdk5 exists in three forms: free monomeric Cdk5, a heterodimeric complex of Cdk5-p25, and multi-protein complexes of Cdk5-p35 (Lee *et al*., 1996a; Rosales *et al*., 2000). As revealed by protein fractionation, Cdk5-p35 exists as large molecular complexes of more than 670 kDa in brain extracts. Accumulating evidence implies that Cdk5-p35 is a multifunctional enzyme that exists in many cellular protein complexes. Consistently, an increasing number of proteins have been reported to associate with Cdk5-p35, providing important clues on the physiological function of Cdk5 as discussed below.

1.3.1 Regulation of Cdk5

The association with a cyclin is essential in activating Cdks. However, Cdk5 activity has not been found to associate with any cyclin. Instead, p35 and p39 were found to be the two specific activators of Cdk5. Although p35 and p39 have little sequence similarity to any cyclin, studies by computer modeling and mutagenesis suggested that p35 might adopt a cyclin-like tertiary structure (Tang *et al*., 1997; Chou *et al*., 1999; Lim *et al*., 2001). Recently, these predictions were further established by the crystallization of a Cdk5-p25 complex (Tarricone *et al*., 2001).

Members of the Cdk family are also regulated by at least three distinct phosphorylation/dephosphorylation events (Fig. 3). Phosphorylation of Cdk1 and Cdk2 at Thr14 and Tyr15 by the dual-specificity kinases Wee1, Myt1 and Mik1 inhibits their activities (Mueller *et al*., 1995b; Mueller *et al*., 1995a; Watanabe *et al*., 1995). In contrast, phosphorylation of Thr160 in the T-loop of Cdk2 (or Thr161 of Cdk1) by the Cdk-activating kinase (CAK) is necessary for its maximal activation (Gu *et al*., 1992). Although Thr14 and Tyr15 are conserved and Thr160 in Cdk2 is conservatively substituted with Ser159 in Cdk5 and their surrounding sequences are highly homologous to those of the authentic Cdks, Cdk5 appears to adopt regulatory mechanisms distinct from those of the classical Cdks at these three phosphorylation sites. The Thr14 and Tyr15 sites in Cdk5 are not phosphorylated by Wee1 *in vitro* (Poon *et al*., 1997). Moreover, Tyr15 of Cdk5 can be phosphorylated by a cytosolic tyrosine kinase *c*-Abl, and such phosphorylation is facilitated by the association of Cdk5 with Cables, an Abl-binding protein. Surprisingly, the phosphorylation of Cdk5 at Tyr15 is stimulatory and enhances Cdk5 kinase activity (Zukerberg *et al*., 2000). In addition to *c*-Abl, Fyn, which is a member of the Src family of tyrosine kinases, is the

other enzyme observed to catalyze the stimulatory Tyr15-phosphorylation of Cdk5 (Sasaki *et al*., 2002). Cdk5 phosphorylation by Fyn is necessary for semaphorin-3A-induced neuronal growth cone collapse (Sasaki *et al*., 2002). Lastly, the phosphorylation of Cdk5 at Ser159, which occupies a position equivalent to the Thr160 site in the conserved T-loop of Cdk2 (Thr161 of Cdk1), not only is dispensable for but also dampens the activation of Cdk5 (Tarricone *et al*., 2001). The crystal structure of Cdk5-p25 revealed that the interaction between the regulatory subunit alone is sufficient to stretch the activation loop of unphosphorylated Cdk5 into a fully extended active conformation, analogous with the phosphorylated Cdk2-cyclin A complex (Tarricone *et al*., 2001).

Another mode of Cdk regulation involves a diverse family of inhibitory proteins (CKIs) that bind Cdks or Cdk-cyclin complexes to inhibit the Cdk activity (Li and Blow, 2001). The initial evidence of the existence of Cdk5 inhibitors comes from the biochemical separation of Cdk5 complexes in brain extracts. The Cdk5-p35 macromolecular complexes are neither enzymatically active nor activable by the addition of a truncated form of p35 (Lee *et al*., 1996a). Furthermore, the kinase activity was recovered when the Cdk5-p35 complexes was further fractionated by size-exclusion chromatography in the presence of 10% ethylene glycol, suggesting that an inhibitor(s) could be dissociated from the complexes under this stringent condition. Interestingly, Cdk5 is not regulated by any of the known CKIs, such as members of the INK and CIP/KIP families of inhibitors (Lee *et al*., 1996b), confirming the distinct structural and regulatory properties of Cdk5-p35 in the macromolecular complexes. A few protein candidates have been reported to be endogenous inhibitors of Cdk5. C42, which is a p35-binding protein, has been shown

to specifically inhibit the activation of Cdk5 by p35 (Ching *et al*., 2002). The inhibitory domain of C42 was mapped to a region of 135 amino acids, which is conserved in Pho81, a yeast protein that inhibits the yeast cyclin-dependent protein kinase Pho85 (yeast functional homologue of mammalian Cdk5) (Huang *et al*., 2001). DNA binding protein dbpA and ribosomal protein L34 are two other reported inhibitors of Cdk5 (Moorthamer *et al*., 1999; Moorthamer and Chaudhuri, 1999). They were identified in a yeast two-hybrid screen as Cdk5-binding proteins. In addition to the inhibitors, the nuclear protein SET was found to enhance Cdk5 activity upon its physical association with Cdk5-p35. The SET protein binds p35 in its N-terminal region, which is lacking in p25, and therefore does not affect the activity of Cdk5-p25, suggesting specific modulation of the Cdk5-p35 activity in nucleus (Qu *et al*., 2002).

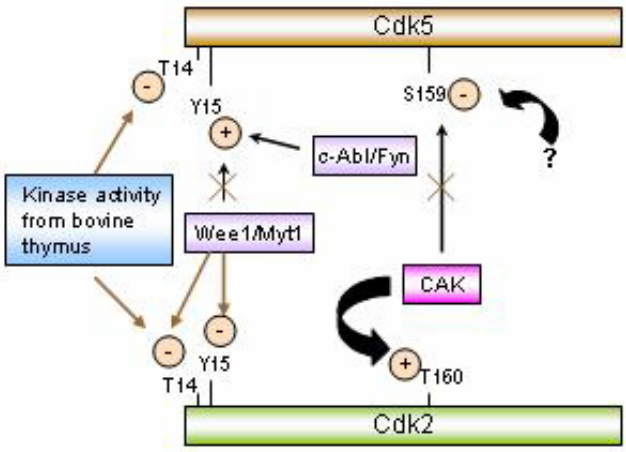

FIG. 3. Regulation of Cdk5 and Cdk2 by phosphorylation. Phosphorylation of Thr160 on Cdk2 by Cdk-activating kinase (CAK) results in a 200-fold activation of the kinase. CAK does not phosphorylate Cdk5 on the equivalent Ser159. Structural analysis of the p25–Cdk5 complex indicates that phosphorylation on Ser159 would be inhibitory. The dual-specificity kinases Wee1 and Myt1 phosphorylate Cdk2 on Thr14 and Tyr15, resulting in an inhibition of kinase activity. Neither of these kinases phosphorylates Cdk5. However, Tyr15 of Cdk5 is phosphorylated by *c*-Abl and Fyn, which stimulate its kinase activity. A kinase activity purified from bovine thymus can phosphorylate Thr14 on both Cdk5 and Cdk2, resulting in inhibition of activity. Green and red arrows represent stimulatory and inhibitory phosphorylation events, respectively.

1.3.2 Physiological roles of Cdk5 and its mediated functions

Over the last decade, a number of proteins have been identified to act as direct substrates of Cdk5 (Table B), providing a good deal of knowledge on the biological roles of Cdk5 in brain. It appears that Cdk5 acts prominently in many essential cellular processes, including cytoskeletal dynamics, cell adhesion, axonal guidance, dopaminergic signaling and synaptic membrane functions.

1.3.2.1 Cdk5 in cytoskeletal dynamics and microtubule-based transport

A body of evidence has implicated an indispensable role of the Cdk5-p35 kinase in axonal guidance, cell motility and neurite outgrowth. Overexpression of p35 in cultured neurons induces the formation of longer neurites whereas inhibition of the Cdk5 activity or expression of a dominant negative form of the kinase prevents neurite outgrowth (Nikolic *et al*., 1996). Cdk5-p35 colocalizes with F-actin, Rac and p21-activated kinase (PAK1) in the periphery of growth cones. Since Cdk5-p35 down-regulates PAK1 kinase activity by phosphorylating it at Thr212, it has therefore been proposed to have a role in regulating actin repolymerization and therefore growth cone dynamics (Rashid *et al*., 2001).

Another group of Cdk5 substrates are the intermediate and heavy chains of neurofilament proteins (NFM and NFH, respectively). NFM and NFH contain many KSP (Lys-Ser-Pro) repeats in their long carboxyl terminal tails. Phosphorylation at the KSP sites occurs during the axonal transport of the neurofilaments and the phosphorylation is required in maintaining axonal morphology. Cdk5 was originally isolated from the brain as a neurofilament kinase to catalyze the KSP phosphorylation at the tail region of NFM and NFH (Lew *et al*., 1992b). Phosphorylation of these

domains by Cdk5 reduces its association with microtubules, as well as retarding the axonal transport of these proteins (Wada *et al*., 1998). *In vitro*, NFH phosphorylated by Cdk5 displayed the same electrophoretic motility shift as that of natively phosphorylated NFH (Grant *et al*., 2001). Moreover, p35 associates with NFM and NFH in a region adjacent to the KSP-rich domains, suggesting a role of p35 in docking Cdk5 to the substrates (Qi *et al*., 1998).

It has been found that Cdk5 associates with microtubules and can be co-purified with microtubules from bovine brain (Ishiguro *et al*., 1992; Sobue *et al*., 2000). Several microtubule-associated proteins (MAPs), such as tau and MAP1B, are substrates of Cdk5. Phosphorylation of tau by Cdk5 abolishes the ability of tau to bind microtubules and therefore its ability to promote microtubule assembly (Wada *et al*., 1998). In addition to the phosphorylation of the MAPs to mediate microtubule stability, Cdk5 has been implicated to play a role in regulating dynein-mediated axonal transport. Nudel is a cytoplasmic dynein-associated protein that is highly expressed in the brain. Cdk5 can phosphorylate Nudel *in vitro* and *in vivo*, and this is of importance since the introduction of a non-phosphorylatable mutant of Nudel into the cultured neurons led to axonal swelling, analogous to the disruption of dynein function in *Drosophila* neurons (Liu *et al*., 2000).

1.3.2.2 Cdk5 in synapses and focal adhesion sites

Cdk5, p35 and p39 are present in the subcellular fractions enriched for synaptic membrane and they are localized to the pre- and post-synaptic compartments (Humbert *et al*., 2000b; Niethammer *et al*., 2000), indicating that they may be involved in synaptic functions. Indeed, several synaptic proteins have been identified as Cdk5 substrates, including Munc 18, synapsin I and amphiphysin, which are

proteins implicated in synaptic vesicle exocytosis (Fig. 4) (Fletcher *et al*., 1999; Matsubara *et al*., 1996; Floyd *et al*., 2001). Phosphorylation of Munc 18 by Cdk5 results in disassembly of the Munc 18-syntaxin I complex, implying a role for Cdk5 in modulating neurosecretion (Shuang *et al*., 1998). Most recently, interesting findings by Tan and co-workers have established that Cdk5 has a role in synaptic vesicle endocytosis (Tan *et al*., 2003). Cdk5 phosphorylates dynamin I *in vitro* as well as *in vivo* at the nerve terminals of neuronal cells to facilitate synaptic endocytosis. Roscovitine, an antagonist of Cdk5 activity, blocks the rephosphorylation of dynamin I after repolarization of the synaptosomes. Furthermore, phosphorylation by Cdk5 also increases the GTPase activity of dynamin I (Tan *et al*., 2003). Hence, Cdk5 may play a major role at synapses since multiple Cdk5 substrates are involved in the synaptic vesicle recycling. In a yeast two-hybrid screen, α-actinin 1 and the α-subunit of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKIIα) were identified as p35- and p39-interacting proteins (Dhavan *et al*., 2002). Either of these two proteins forms a complex with Cdk5 through the interaction with p35 or p39 and these interactions potentially localize Cdk5 to the post-synaptic density where it may play a role contributing to synaptic plasticity, memory and learning.

N-cadherin is a member of the transmembrane molecules that promote cell adhesion by their calcium-dependent homophilic interactions. The cytoplasmic tail of cadherins interacts with α- and β-catenin to anchor the cadherins to the actin cytoskeleton. Cdk5-p35 is associated with β-catenin and controls the *N*-cadherin/β-catenin-mediated cell adhesion through the phosphorylation of β-catenin (Kwon *et al*., 2000; Kesavapany *et al*., 2001). Additionally, phosphorylation of β-catenin by Cdk5

has also been shown to affect its binding to presenilin, which is a pathological molecule of the Alzheimer's disease (AD) (Kesavapany *et al*., 2001).

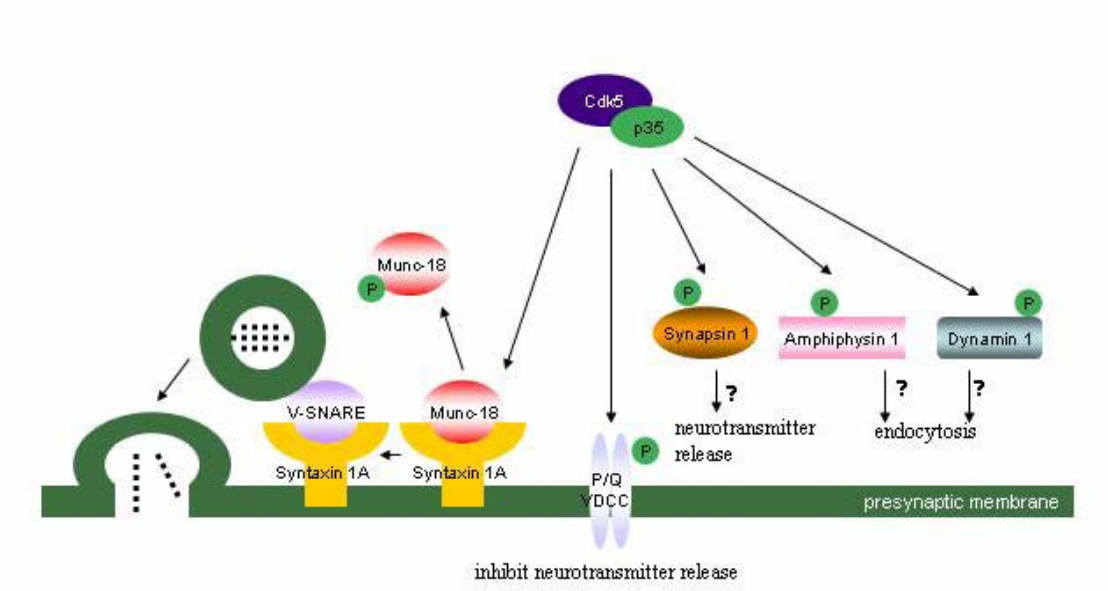

FIG. 4. Cdk5 in neurotransmitter release and endocytosis at the presynaptic terminals. In the absence of Cdk5, Munc-18 binds syntaxin 1A, interfering the interaction between syntaxin 1A and v-SNAREs, which is required for synaptic vesicle to gain competency for membrane fusion. Upon phosphorylation by Cdk5, Munc-18 is dissociated from syntaxin, allowing the syntaxin 1A/v-SNAREs interaction and leading to enhanced neurotransmitter release. In contrast, Cdk5 inhibits neurotransmitter release through phosphorylation of P/Q type voltage-dependent calcium channel (VDCC), resulting in the dissociation of VDCC from SNAREs, which attenuates the efficiency of neurotransmission. Cdk5 also phosphorylates synapsin I and amphiphysin1/dynamin1, which are potentially involved in neurotransmitter release and endocytosis, respectively, although the physiological significance remains to be determined.

1.3.2.3 Cdk5 in neurosignaling

Accumulating evidence suggests that Cdk5 is involved in many of the neuronal signal transduction pathways. DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, M_r of 32 kDa) is a Cdk5 substrate that plays a key role in dopamine signaling occurring in the dopaminoceptive neurons. When DARPP-32 is phosphorylated by the cAMP-dependent protein kinase (PKA), it becomes a potent inhibitor of protein phosphatase 1 (PP1). It appears that the Cdk5 phosphorylation of DARPP-32 transforms it into an inhibitor of PKA, exerting an opposing effect on dopamine signaling (Bibb *et al*., 1999; Greengard *et al*., 1999). In the MAPK

(mitogen-activated protein kinase) and JNK (*c*-Jun N-terminal kinase) pathways, Cdk5 is associated with JNK-3 where it inhibits the JNK-3 activity by phosphorylating JNK-3 at Thr131 to mediate neuronal apoptosis (Li *et al*., 2002). Meanwhile, Cdk5 down-regulates the MAPK signaling by phosphorylating MEK1 (MAPK kinase 1) at Thr286 and thereby inhibiting its activity (Sharma *et al*., 2002). In addition, Cdk5 has been shown to mediate the activities of the P/Q-type voltage-dependent calcium channel and the *N*-methyl-*D*-aspartate (NMDA) class of glutamate receptors through a direct phosphorylation modification (Tomizawa *et al*., 2002; Li *et al*., 2001).

1.3.2.4 Cdk5 in transcriptional machineries

Recent studies have pointed to the localization of Cdk5-p35 in the nucleus where it may play a role in transcriptional regulation. In an early report, Cdk5 was found to bind and phosphorylate the retinoblastoma susceptibility gene product (pRb) (Lee *et al*., 1997). p53 is a phosphorylation-regulated transcription factor that plays a pivotal role in cell cycle progression and cell death. Cdk5 is able to modulate the p53 transcriptional activity through the direct phosphorylation of p53 as well as the elevation of p53 expression in the cells (Zhang *et al*., 2002b). More recently, myocyte enhancer factor 2 (MEF2) was identified as a Cdk5 substrate (Gong *et al*., 2003). Members of the MEF2 family are transcription factors that play critical roles in diverse cellular processes including neuronal survival. Phosphorylation of MEF2 by Cdk5 results in the inhibition of the MEF2 transactivation activity, while MEF2 mutants that are resistant to the Cdk5 phosphorylation rescue neurons from neurotoxin/Cdk5-induced apoptosis, suggesting MEF2 phosphorylation by Cdk5 as a

part of the molecular mechanism by which neurotoxin/Cdk5 mediates apoptosis (Gong *et al*., 2003).

1.3.3 Molecular organization of Cdk5 complexes

A body of evidence has suggested that Cdk5-p35 kinase shows high affinity binding to specific cellular proteins. To date, there are about 30 or more proteins with diverse functions that have been identified to associate with Cdk5-p35 or Cdk5-p39 (Table A). Cdk5 itself appears to bind directly with a smaller subset of these proteins, which include Cables, tau, PP1, dbpA and ribosomal protein L34. Many of the other proteins interact with Cdk5 via p35 or p39, suggesting that p35 and p39 are not only acting as the activators of Cdk5 but are also important mediators of the Cdk5 function. In addition, a number of proteins have been established to be substrates of Cdk5 (Table B). Identification of these substrates, as well as the Cdk5/p35- and Cdk5/p39-associated proteins, has revealed numerous important functional and regulatory properties of Cdk5.

By immunocytochemistry and cellular fractionation analysis, Cdk5 and p35 proteins were detected throughout the cells with a much lower level in the nucleus (Qu *et al*., 2002). p35 is enriched in the membrane fraction and the association of Cdk5-p35 with the plasma membrane is directed by the myristoyl moiety linked to the N-terminal glycine of p35 (Patrick *et al*., 1999; Patzke and Tsai, 2002). Moreover, Cdk5-p35 extracted from a membrane preparation of rat brains exhibited the biochemical property of large molecular complexes (R. Gao and R.Z. Qi, *unpublished observation*). Conceivably, the membrane localization is important for Cdk5 to exert many of its physiological effects and some of its substrates are likely to be membrane-integral or membrane-associated proteins. Additionally, an active Cdk5-p35 kinase has been shown to be present in Golgi membranes, where it associates with a detergent-insoluble fraction that contains actin (Paglini *et al*., 2001). Suppression of

the Cdk5 activity blocks the formation of membrane vesicles from the Golgi apparatus, possibly suggesting a role for Cdk5-p35 in membrane trafficking. Further, Cdk5-p35 may be involved in the reorganization of actin in the growth cone and on the Golgi membrane during neurite outgrowth.

Under certain conditions, p35 is converted to p25 by a proteolytic cleavage, resulting in the truncation of the smaller N-terminal fragment of p35, commonly known as p10 (Kusakawa *et al*., 2000; Lee *et al*., 2000). The transformation of p35 to p25 appears to exclude it from most of the components of the Cdk5-p35 macromolecular complexes, implying that the p10 region of p35 might be required for interaction with other proteins. Indeed, deletion analyses have provided proof of specific and direct interaction between p10 and at least one p35-interacting protein (Qu *et al*., 2002). Another region of 26 amino acids spanning residues 145 to 170 of p35 has also been identified to contain the binding site for a few of the identified p35-binding proteins (Wang *et al*., 2000; Cheng *et al*., 2002). This short stretch is proximal to the N-terminal boundary of the Cdk5-binding and activating domain in p35, and contains an amphipathic α-helix (Chin *et al*., 1999). Further evidence from the interaction studies indicates that the hydrophilic face of the helix is involved in the interaction with the binding proteins while the hydrophobic face is involved in the association with Cdk5 (Wang *et al*., 2000). This unique feature of the p35 structure may be necessary to support a number of other functions when bound to its interacting proteins, in addition to kinase activation.

1.3.3.1 Methods used in isolating protein-interacting partners

Various methods have been described in an increasing number of reports over the last few years to uncover the protein components of Cdk5 macromolecular complexes in the brain. Using the yeast two-hybrid system, a number of proteins have been found by screening mammalian brain libraries that specifically interact with Cdk5, p35, or p39 (Dhavan *et al*., 2002; Ching *et al*., 2000; Moorthamer and Chaudhuri, 1999). The yeast two-hybrid interaction screen is a sensitive method with which transient or weak interactions can be detected. However, it is not ideal for the screening of membrane-associated proteins, and proteins which are not able to translocate into the nucleus. Moreover, the usage of this method is also limited to the detection of dimeric protein complexes and, therefore, is not particularly effective for identifying multimolecular complexes. Further, some interactions may require certain post-translational modifications of their protein partners. However, such modifications may not occur in yeast.

Another major method to identify protein-protein interactions at a high throughput and at a proteome-wide scale is the biochemical isolation of protein complexes from animal tissues or cultured cells (Ho *et al*., 2002; Gavin *et al*., 2002). In several laboratories, biochemical isolation of Cdk5 and p35-associated proteins was conducted using affinity chromatography media prepared by coupling antibodies recognizing Cdk5 or p35 or by coupling recombinant proteins derived from Cdk5 and p35 to agarose beads (Qu *et al*., 2002). The identity of isolated interacting proteins from rat brain extracts was established using mass spectrometry. An advantage of this method is the ability to isolate protein complexes and interacting proteins in their native states. It is particularly useful in identifying indirectly interacting proteins,

since multiprotein complexes can be isolated at ease. However, it is less favorable with proteins having weak or transient interactions. Therefore, this approach would be a good complement to the yeast two-hybrid interaction screen.

Another favored procedure of isolating components of a multiprotein complex is the use of combinatory chromatography procedures (Luo and Roeder, 1995; Rosales *et al*., 2000). In general, total lysate, prepared from cultured cells or tissue in a preferred buffered system, is subjected to differential chromatography procedures where the elution profile of the protein of interest (bait protein) is followed commonly by its immunoreactivity. Fractions from a previous chromatography separation were pooled, dialyzed and enriched before subjected to a subsequent chromatography procedure. Samples of near homogeneity were then analyzed by SDS-PAGE and subsequently isolated protein bands were identified by protein analysis methodologies. Likewise, this protocol allows the isolation of interacting partners in a native state where indirect interacting molecules of a protein complex can be identified. However, analyzing proteins of transient interaction is less favorable in this context.

1.4 Microtubule Dynamics

Microtubules, together with actins and intermediate filaments, are the key components of the eukaryotic cytoskeleton. Microtubule is an essential component of the cellular cytoskeleton providing mechanical support for the shape maintenance of cell and serves as tracks along which motor proteins move organelles and transport vesicles from one part of the cell to another. Microtubules are long, filamentous, tube-shaped protein polymers that are crucial in the development and maintenance of cell shape, in the transport of vesicles, mitochondria and other components throughout cells, in cell signaling and in cell division and mitosis. To perform these functions, a cell must control the assembly and orientation of its microtubule cytoskeleton. Microtubule arrays in cells are generally dynamic, capable of assembly, disassembly and rearrangement on a time scale of seconds to minutes. Microtubule dynamics *in vivo* are based on intrinsic dynamic properties of the polymers themselves, being determined by biochemical properties of the microtubule building block, the tubulin αβ heterodimer. Tubulin is able to interact with a bewildering number of proteins and small molecules such as nucleotides and drugs. Furthermore, tubulin is a GTPase. GTP hydrolysis during tubulin assembly is apparently a phenomenon of central importance for microtubule physiology.

Tubulins are ubiquitously expressed in eukaryotes. α- and β-tubulin monomers are proteins of about 450 amino acids each and are about 50% identical at the amino acid level (Wade and Hyman, 1997). Each monomer has a M_r of about 50 kDa and binds a GTP molecule that is non-exchangeable in α-tubulin and exchangeable in β-tubulin. GTP from β-tubulin is required for microtubule assembly

and its hydrolysis follows addition of a dimer to the microtubule end, upon which it becomes non-exchangeable within the microtubule. The structure of the tubulin dimer has been solved by electron crystallography of the two-dimensional, crystalline sheets of tubulin that form in the presence of zinc ions (Nogales *et al*., 1998). This atomic model of tubulin provides structural information and gives a picture of the properties of tubulin and microtubules at the molecular level (Nogales *et al*., 1999).

The organization of α- and β-tubulin heterodimers in the microtubule lattice is polarized, and this feature results in structural and kinetic differences at the microtubule ends. The faster growing end (named the plus end) has the β-tubulin subunit of each heterodimer exposed, whereas the slower growing end (named the minus end) has the α-tubulin subunit exposed. *In vivo*, the minus end of the microtubule is associated with the centrosome. It is localized near the center of the cell, whereas the plus end is peripheral. Once assembled, tubulin addition to and loss from the existing microtubules occurs only at the microtubule ends.

In vitro, the aggregation of purified tubulin to form microtubules can be induced in a variety of conditions. Two factors play an important role. First, microtubules do not form at low temperatures. Therefore, tubulin aggregation is generally induced by warming up tubulin solutions to 30-37°C. Secondly, tubulin is incorporated into microtubules as a complex with GTP. Following warming and GTP addition to the soluble tubulin dimers, tubulin assembly proceeds and one of the widely used methods used to follow microtubule assembly has been the turbidity measurements. Microtubule suspensions are turbid and cause an apparent increase in absorption at 340-350 nm. This property is used to measure the total mass of

assembled microtubules and gives information about the behavior of the whole microtubule population (Fig. 5).

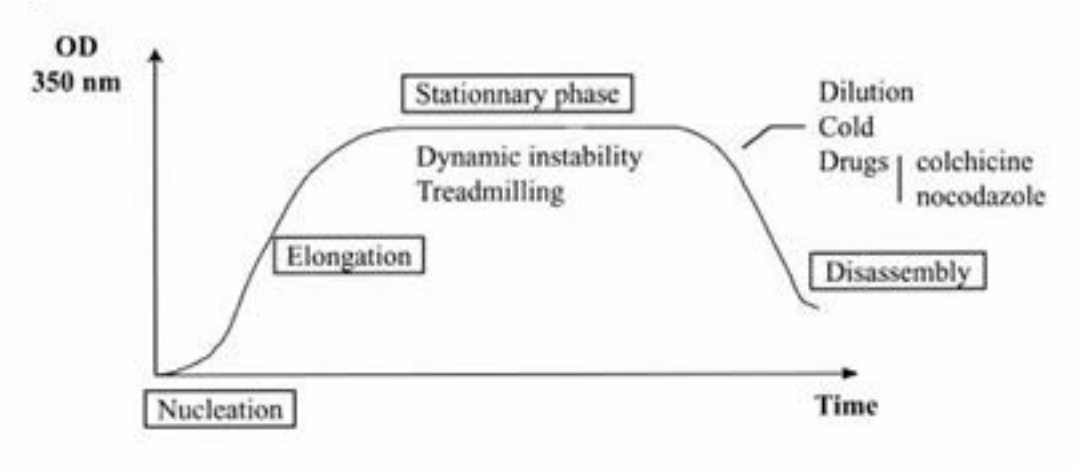

FIG. 5. Microtubule assembly. Various phases of microtubule assembly as seen in turbidmetry. Modified from (Valiron *et al*., 2001).

As measured by turbidity, the first phase of assembly is usually referred to as the nucleation phase. During this phase, poorly defined microtubule seeds are formed. Microtubule seeds do not convert into microtubules below a tubulin threshold concentration defined as critical concentration, which essentially depends on experimental conditions. This critical concentration depends on experimental assembly conditions. The second phase is that of elongation where tubulin dimers are added at the end of microtubule. During this phase, tubulin dimers add at the end of microtubule. The third phase of assembly is that of steady-state. Analysis of steady-state microtubules has demonstrated that a fixed proportion of tubulin is in the assembled forms whereas another tubulin pool remains soluble. The concentration of free tubulin dimers at steady state varies with buffer conditions and corresponds to the so-called tubulin critical concentration for microtubule assembly. Tubulin assembly has two main features: the microtubules formed are intrinsically labile and, at steady state, the microtubule system remains out of equilibrium. Microtubules are readily

disassembled by temperature drop below 10-15°C, removal of GTP or dilution of microtubule suspensions and subsequent decrease of the free tubulin concentration. Microtubule disassembly can also be triggered by adding specific drugs to a microtubule solution such as colchicine, vinblastine or nocodazole. Drugs like colchicine and vinblastine poison tubulin assembly onto microtubule ends and thereby induce microtubule disassembly. Nocodazole acts by sequestering tubulin dimers, with which it forms an inactive complex. Hence, the free tubulin concentration decreases, and when it drops below the critical concentration, microtubules disassemble.

At steady state, microtubules remain out of equilibrium. Continuous GTP hydrolysis and thereby energy consumption is required for the maintenance of steady-state microtubules, which apparently use energy to generate active exchanges between their constitutive subunits and the tubulin molecules of the soluble pool. Two mechanisms are thought to account for such tubulin exchanges. The first has been known treadmilling. Treadmilling relies on a particular property of microtubules: at steady state, microtubule ends behave differently (Margolis and Wilson, 1978). The plus end continuously incorporates new tubulin dimers, whereas the minus end continuously looses them. When microtubule ends are not anchored at a fixed position, treadmilling results in an apparent microtubule migration that actually reflects the propagation of a wave of tubulin assembly-disassembly. When microtubule ends are kept stationary, treadmilling generates an apparent flux through the polymers of tubulin molecules traveling from the plus ends towards the minus ends (Margolis and Wilson, 1981). The second mechanism through which steady state microtubules exchange their constitutive tubulin with soluble tubulin relies on another

property known as dynamic instability (Mitchison and Kirschner, 1984). In this model, although a population of microtubules exhibits a bulk steady state, an individual microtubule never reaches an equilibrium length but persists in prolonged phases of assembly and rapid phases of disassembly that interconvert frequently. Transit from slow assembly to fast disassembly is referred to as 'catastrophe' and transition from disassembly to assembly as 'rescue'. Moreover, microtubules sometimes pause for a period of time, during which their length remains constant (Walker *et al*., 1988).

Microtubule dynamics *in vivo* share fundamental features with microtubule dynamics *in vitro*. Cellular microtubules are highly dynamic, and both dynamic instability and treadmilling have been observed *in vivo*. Cellular microtubules exhibit a more rapid assembly rate and higher transition frequencies than in vitro microtubules formed from pure tubulin (Cassimeris, 1993). In addition, microtubule dynamics *in vivo* are regulated by many MAPs, and these are usually classified into two main groups: proteins that stabilize microtubules and proteins that destabilize microtubules (Desai and Mitchison, 1997; Mandelkow and Mandelkow, 1995). The former includes several mammalian MAPs that promote tubulin assembly and stabilize microtubules once they are formed (often referred as structural MAPs), although they do not prevent disassembly (Hirokawa, 1994). Members of this family include the neuronal proteins tau and MAP2, which are localized to the axon and the dendrites, respectively, and MAP4, which is present in all non-neuronal vertebrate cells. In all three proteins, the microtubule binding domain is contained within the C-terminus, where the N-terminus domain protrudes from the microtubule surface. The effect of MAPs on microtubule stability often depends on phosphorylation, and all

structural MAPs have been shown to be *in vitro* substrates for several protein kinases. For instance, microtubule-affinity-regulating kinases have been described as mammalian serine/threonine kinases that phosphorylate the tubulin binding domain of MAPs, cause their detachment from microtubules and therefore increase microtubule dynamics (Drewes *et al*., 1998).

On the other hand, gene targeting experiments have been used to establish that these structural MAPs are necessary for the proper development of the nervous system (Harada *et al*., 2002; Teng *et al*., 2001; Takei *et al*., 2000; Takei *et al*., 1997; Harada *et al*., 1994). For instance, *MAP1B*$^{-/-}$ mice showed a slightly decreased brain weight and a delay in the development of the nervous system. Hence, MAP1B is not essential for survival, but it is essential for the normal time course of brain development (Takei *et al*., 1997).

More recently, another group of microtubule-associated proteins that is able of inducing much higher microtubule stability has been described and these proteins include Lis1 and doublecortin, BPAG1 and STOP proteins. Lis1 is a subunit of the platelet-activating factor acetylhydrolase, and *in vitro* experiments showed that the protein interacts with tubulin and microtubules. It reduces microtubule catastrophe events, and this leads to an increase in the maximum length of the microtubules (Sapir *et al*., 1997). Doublecortin associates directly with microtubules *in vitro* and *in vivo* and stabilizes them. Its sequence does not contain any known microtubule binding domain, suggesting that it defines a new microtubule-binding structure (Horesh *et al*., 1999). In transfected cells, BPAG1 binds and stabilizes microtubules, which become resistant to depolymerzing agents, including cold (Yang *et al*., 1999).

Section 2

Materials and Methods

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Colchicine, PMSF, nerve growth factor (NGF), common chemicals and reagents were purchased from Sigma Chemical Co (St. Louis, MO). Roscovitine was purchased from Calbiochem (La Jolla, CA), while Complete™ protease inhibitor cocktail was from Roche Molecular Biochemicals (Mannheim, Germany). Cell culture reagents as well as IPTG were purchased from Invitrogen (Carlsbad, CA), while restriction enzymes were from New England Biolabs (Beverly, MA). The one-step RT-PCR kit was purchased from Qiagen GmbH (Hilden, Germany) while PfuTurbo® DNA polymerase was from Stratagene (La Jolla, CA). Protein A sepharose fast flow and Glutathione sepharose 4B (GSH-sepharose) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), while nickel-NTA beads were from Qiagen (Hilden, Germany). The enhanced chemiluminescence (ECL) kit was purchased from Pierce (Rockford, IL). Purified tubulin (>99% pure and MAP-free), rhodamine-labeled tubulin and the microtubule-associated protein spin-down kit were purchased from Cytoskeleton Inc (Denver, CO). siRNA duplexes used were synthesized by Dharmacon Inc. (Lafayette, CO). CK2 holoenzyme protein was from New England Biolabs, while CK2α and CK2β were purchased from Calbiochem. Effectene^{TM} transfection reagent was purchased from Qiagen GmbH, Lipofectamine reagent from Invitrogen and TransIT-TKO reagent was from Mirus (Madison, WI). Nitrocellulose and PVDF membranes were from Amersham Pharmacia Biotech and Millipore, respectively. [γ-^{32}P] dATP was purchased from Amersham Pharmacia Biotech. The vectors, pSG5 containing chicken CK2α (pSG5-CK2α) and CK2αK68A (pSG5-CK2αK68A) cDNA used for transient transfection were kindly

provided by Claude Cochet (Leroy *et al*., 1997). Tau protein expression vector was from Michel Goedert (Goedert *et al*., 1995).

2.1.2 Cell lines

To reduce the danger of contamination, all works were carried out under sterile conditions in a laminar flow hood. Work area surfaces were readily cleaned and all the items used in cell culture were purchased as sterile, disposable items. The cell lines used in this study include COS-7 monkey kidney cells, HeLa human cervical carcinomas cells, 293T human kidney epithelial cells, PC-12 rat pheochromocytoma cells and SH-SY5Y neuroblastoma cells. COS-7, HeLa, 293T and SH-SY5Y cells were maintained in DME low glucose (1000 mg/l) medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin and 100 mg /ml streptomycin. PC-12 cells were maintained in DME high glucose (4500 mg/l) medium supplemented with 10% horse serum, 5% FCS, 1% L-glutamine, 100 U/ml penicillin and 100 mg /ml streptomycin. All cells were kept at 37°C under a partial CO_2 pressure of 5%. Passaging, thawing and freezing of cells were performed using standard protocols (Freshney, 1992). PC-12 cells were induced with 100ng/ml NGF for 48-96 hrs before studies.

2.1.3 Antibodies

Monoclonal antibodies against α- and β–tubulin were purchased from Sigma Chemical Co (St. Louis, MO). The following polyclonal antibodies: anti-p35 (C19 and N20), anti-Cdk5 (C8), anti-CK2α (C18 and H-286), anti-CK2β (C19), anti-β-tubulin (H-235), anti-actin (I-19) and anti-*c*-Myc; and monoclonal antibodies: anti-Cdk5 (DC17), anti-CK2α, anti-CK2β and anti-GST were purchased from Santa Cruz

Biotech (Santa Cruz, CA). CK2α and CK2β monoclonal antibodies were also purchased from Transduction Laboratories (Lexington, KY). Polyclonal antiserum against CK2α was purchased from Calbiochem. The immobilized *c*-Myc monoclonal antibody was purchased from Convance Inc (Princeton, NJ). Monoclonal anti-HA antibody was from Cell Signaling Technology (Beverly, MA), while anti-*c*-Myc and anti-His monoclonal antisera were purchased from Clontech (Palo Alto, CA). Anti-V5 monoclonal antibody was purchased from Invitrogen. The secondary antibodies are Fluor594/Fluor488 conjugated goat anti-mouse IgG, Fluor594/Fluor488 conjugated anti-rabbit IgG and Fluor488 conjugated donkey anti-goat IgG from Molecular Probes (Eugene, OR). Secondary HRP-conjugated anti-IgG antibodies for immunoblotting were from Amersham Life Sci. (Chicago, USA).

2.2 Experiment Procedures

2.2.1 Plasmid constructions

Various fragments of p35 were engineered by PCR into the vectors pGEX2T or pGEX4T (Amersham-Pharmacia) for preparation of GST fusion proteins. They are p10 (residues 1-98 of p35), p16 (residues 1-149 of p35), p35 (53-149) and p35 (150-307). The constructs of GST-Cdk5 and GST-p25, which includes residues 99-307 of p35, have been reported previously (Qi *et al*., 1995). The coding sequence of Cdk5 was cloned by PCR into the pET32a vector for the expression of His-Cdk5 protein. The expression construct of p35-His was generated by insertion of a PCR sequence of p35 into pQE12 (Qiagen). The resulting plasmid, p35/pQE12, allows expression of p35 with a C-terminal His-tag. DNA fragment encoding p35-His was restricted from the pQE12 construct and inserted into pGEX2T vector for expression of GST-p35-His recombinant protein. Cdk5 and p35 were also engineered into pcDNA3.1 (Invitrogen) and pCI-neo (Promega) for protein expression in mammalian cells.

The coding sequences of chicken CK2α and its kinase-inactive mutant (CK2αK68A) were subcloned into pGEX4T, pET32a (Novagen) and pDneo-myc (Tang *et al*., 2001). The full-length sequence of human CK2β was cloned by RT-PCR and inserted into pQE30 (Qiagen). CK2α/CK2αK68A and CK2β were engineered by PCR into the bicistronic vector pBudCE4.1 (Invitrogen). All clones were verified after sequencing entirely in both directions and propagated in *Escherichia coli* strain XL1-blue or DH5α. All plasmids were purified using miniprep or midiprep kit (QIAGEN) for subsequent use in transfection experiments.

2.2.2 Recombinant protein preparation

The GST fusion proteins were prepared as described previously (Qi *et al*., 1995). Briefly, GST-tagged proteins were individually expressed in E. coli BL21 (DE3). The host cells were cultured in 1 liter of LB medium containing 100 μg/ml ampicillin to an OD_{600} of 0.5-0.8 at 37°C. The synthesis of GST recombinant protein was induced with 0.5 mM IPTG for a subsequent incubation of 3 hrs at 25°C. The cells were harvested by centrifugation at 2000 *g*. The cell pellets were washed with PBS and pelleted again for subsequent purification of the recombinant proteins. The cell pellets were resuspended in 20 ml of 50 mM Tris-HCl (pH 7.4) buffer containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, 0.25 mM PMSF, and protease inhibitor mixture (lysis buffer). All procedures for the purification were carried out at 4°C. The cells were lysed by sonication and centrifuged at 18000 *g* for 30 mins. The supernatant was applied onto a 2 ml column of GSH-sepharose which had been equilibrated with the lysis buffer. The column was sealed and allowed to sit on an end-over-end shaker for 2 hrs. After loading, the column was washed with 10 bed volume of lysis buffer supplemented with 0.5 M NaCl, and 5 bed volume of the lysis buffer without detergent (Buffer L). The GST fusion protein was eluted by 5 mM reduced glutathione in Buffer L and dialyzed in a 25 mM Tris-HCl (pH 7.4) buffer containing 100 mM NaCl, 1 mM EDTA and 1 mM DTT (dialysis buffer).

Expression of His_6-tagged recombinant proteins in bacteria and purification with Ni-NTA beads were performed according to the manufacturer's instruction. Briefly, bacteria expressing the His-tagged protein were cultured, harvested and washed as mentioned above. The cell pellet was resuspended in a sodium phosphate (pH 8.0) buffer containing 150 mM NaCl, 1% Triton X-100, 20 mM imidazole, 10 mM β-mercaptoethanol and protease inhibitor mixture (lysis buffer). The cells were

sonicated, cell extract was harvested by sonication, and the supernatant was applied to a Ni-NTA column. After incubation, the column was washed with 10 bed volume of the lysis buffer without detergent (washing buffer) and eluted with 250 mM imidazole in the washing buffer. Eluted proteins were dialyzed in the dialysis buffer described above. Purification of GST-p35-His was performed by sequential chromatography using GSH-sepharose and Ni-NTA beads, respectively, to yield a purer p35 recombinant protein. Purified proteins were stored in small aliquots at -80°C until used.

2.2.3 Isolation of p35-binding proteins

The whole isolation procedure was carried out at 4°C. Rat brains were homogenized in Buffer A (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 1 mM PMSF, 0.1 mM benzamidine and Complete protease inhibitor cocktail). The crude homogenate was centrifuged at 100,000 g for 1 hr and the lysate was carefully collected. GSH beads coated with 100 μg of GST, GST-p10, GST-p16 or GST-p35-His were mixed with the lysate and incubated for 4 hrs with gentle shaking. The beads were then extensively washed with Buffer B (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1% NP-40, 1 mM PMSF and 0.1 mM benzamidine). After briefly washing the beads with Buffer C (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT and 1 mM EDTA), the coupled proteins were eluted by incubation in Buffer C supplemented with 1 M NaCl (Elution buffer). The beads were eluted twice with the elution buffer. The eluates were combined and 3 volumes of cold acetone were added to precipitate the proteins at -20°C for overnight. The precipitates were washed with cold 70% methanol and

dissolved in SDS-PAGE sample buffer. The samples were resolved by SDS-PAGE and visualized by colloidal Coomassie Blue staining (Pierce).

2.2.4 Mass spectrometry

Protein bands excised from SDS-PAGE gels were destained, reduced with DTT, alkylated with iodoacetamide and then in-gel digested with modified trypsin (Promega, Madison, WI) (Shevchenko *et al*., 1996). The extracted peptides were analyzed on a quadrupole/Time-of-Flight mass spectrometer (QSTAR Pulsar, PE Sciex) equipped with a nanoelectrospray ion source. Protein identities were revealed by querying sequence databases using the MASCOT search engine (http://www.matrixscience.com) with peptide sequence tags generated from tandem mass spectrometry (Wilm *et al*., 1996; Mann and Wilm, 1994). Fig. 1 shows a schematic overview of this procedure.

2.2.5 Biochemical binding assays

For Cdk5/p35 binding assays, 5 µg of GST-tagged proteins were incubated with 10 µg CK2 proteins as indicated in Buffer A for 1 hr at 30°C. The GST-tagged proteins were retrieved by further incubation with GSH-Sepharose for 1 hr at 4°C. The beads were washed four times with 1 ml of the binding buffer. Bound proteins were released by boiling in the SDS-PAGE loading buffer and subsequently analyzed by immunoblotting.

To test tubulin binding, GSH-Sepharose beads (Amersham-Pharmacia) pre-bound with GST, GST-CK2α or the complex of GST-CK2α/His-CK2β were incubated with purified tubulin (>99% pure and MAP-free, Cytoskeleton) for 1 hr at

4°C. After being extensively washed with the binding buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 20 mM $MgCl_2$, 1 mM DTT and 0.1% NP-40), the beads were boiled in the SDS-PAGE sample buffer and analysed by immunoblotting. Antibodies against α- and β-tubulin were from Sigma. The binding of His-tagged proteins with tubulin was performed with Ni-NTA beads in the binding buffer without DTT. In the microtubule binding assay, microtubules, which were pre-assembled with taxol in the PEM buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) supplemented with 1 mM GTP, were incubated with the respective proteins. The samples were subsequently loaded onto a buffered cushion (50% glycerol in the PEM buffer) and centrifuged to pellet the microtubules and associated proteins. The pellet and the supernatant were analyzed by immunoblotting.

2.2.6 Protein size exclusion chromatography

All procedures were carried out at 4°C. Rat brains were homogenized in Buffer B (25 mM Hepes, pH 7.3, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT) with 5μg/ml leupeptin/aprotinin/antipain protease inhibitors cocktail. The crude homogenate was centrifuged at 100, 000 g for 1 hr and the supernatant was carefully collected. Ten milligram of the supernatant was applied to an FPLC Amersham Pharmacia Superdex 200 (HR16/50) gel filtration equilibrated in Buffer A. One ml fractions were collected. Proteins in each fraction were concentrated by overnight acetone precipitation and analyzed by immunoblotting using the antibodies indicated. Molecular weight calibration of the Superdex 200 column was performed under the same conditions using a gel filtration calibration kit from Amersham Pharmacia. The column was calibrated with Dextran blue (2,000 kDa; void volume), thyroglobulin (670 kDa), ferritin (440 kDa), aldolase (158 kDa) and albumin (67 kDa).

2.2.7 Cdk5 *in vitro* kinase assay

The Cdk5 kinase assay was performed according to the method described previously (Qi *et al*., 1995), with modification in accordance to Ching *et al*. (Ching *et al*., 2002). Briefly, the histone kinase activity of the sample was measured in 30 mM MOPS, pH 7.4, and 10 mM $MgCl_2$ at 30°C for 10 mins. The reaction was stopped by the addition of trichloroacetic acid and the incorporation of phosphate into the histone H1 peptide was measured by a scintillation counter. To assess the effect of CK2α/CK2 holoenzyme on Cdk5 activity, proteins were incubated for 30 mins at room temperature before commencement of the assays.

2.2.8 Transient transfections

Transient transfections were performed with Lipofectamine in accordance with the manufacturer's instructions. Briefly, cells were seeded on 100 mm plates overnight before transfection with a total of 12 μg of the various plasmids. Fresh growth medium was replaced after 5 hrs of transfection and cells were further incubated for 24-48 hrs before they were subjected to immunoprecipitation or immunostaining studies.

2.2.9 Immunoprecipitation

For immunoprecipitation, transfected cells or rat brains were lysed in a buffer containing 25mM Tris-HCl, pH 7.4, 100mM NaCl, 1mM EDTA, 0.5% Triton X-100 and protease inhibitor mixture (Buffer C). The lysate were clarified by centrifugation and protein A beads, and used for immunoprecipitation with the indicated antibodies.

After extensive washing with Buffer C, the precipitated proteins were resolved by SDS-PAGE and detected by immunoblotting using the antibodies indicated.

2.2.10 Microtubule assembly

Microtubules were assembled *in vitro* from the purified MAP-free tubulin at 2 mg/ml in the PEM buffer supplemented with 1 mM GTP at 35°C and the turbidity of the solutions was monitored at 340 nm (Gaskin, 1982). CK2 was added at various amounts as indicated to promote assembly. To visualize assembled microtubules, tubulin and rhodamine-labelled tubulin at the ratio of 7:1 were used in the polymerization (Belmont *et al*., 1990). Microtubules were fixed with 0.5% gluteraldehyde and visualized by fluorescence microscopy.

2.2.11 Differential tubulin extraction from intact cells

Differential extraction of tubulin heterodimers and polymers from cells was performed using a protocol described previously (Lieuvin *et al*., 1994). Briefly, cultured cells were lysed with the microtubule-stabilizing buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 10% glycerol and protease inhibitors), which was prewarmed to 35°C, to extract cytosolic soluble tubulin heterodimers and to preserve microtubules (assembled insoluble tubulin polymers). The extract was cleared by centrifugation and the supernatant is designated as the free tubulin fraction. After a brief wash with the microtubule-stabilizing buffer, the pellet was extracted in the microtubule-destabilizing buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 mM $CaCl_2$ and protease inhibitors). The extract was clarified by centrifugation to yield the polymerized tubulin fraction. Both fractions were analyzed by immunoblotting and each band on the blots was quantitated using a

Bio-Rad GS-700 imaging densitometer and analyzed with the Multi-Analyst (version 1.0.1) program (Bio-Rad).

2.2.12 RNAi

The siRNA sequence designed for human CK2α/α' is 5'-CCAGCUGGUAGUCAUCUUGUU-3', which has high sequence homology between both catalytic isoforms but many discrepancies to the corresponding sequence of chicken CK2α/α' (Ulloa *et al*., 1993). Twenty μM of the CK2α/α' siRNA or a scrambled siRNA sequence was used the transfection using *Trans*IT-TKO transfection reagent, according to the manufacturer's instructions. Simultaneous transfection of siRNA and plasmid DNA was done using *Trans*IT-TKO and Lipofectamine concurrently. After transfection, the cells were cultured for 24 hrs before the treatment of 0.2 μM colchicine (Sigma) for 3 hrs. The cells were subjected to the differential extraction of free and polymerized tubulin or immunostaining. For SH-SY5Y cells, transfected cells were incubated for 48 hrs prior to immunoprecipitation and Cdk5 kinase assay.

2.2.13 Immunofluorescence microscopy

At the end of each respective transfection or treatment period, the cells were washed twice in PBS and fixed in PBS containing 4% paraformaldehyde before permeabilization in PBS containing 0.2% Triton X-100. After the blocking wash with 10% goat serum and 0.1% Triton X-100 in PBS, the immunostaining was performed with the indicated primary antibodies, which was followed by the respective secondary antibodies at room temperature. Excess antibodies were removed by extensive washing in PBS. Labelled cells were visualized with a Carl Zeiss Axioplan

microscope equipped with a BioRad MRC-1024 confocal optics system (Coe *et al*., 1999) or a microscope equipped with an AxioCam CCD camera (Carl Zeiss, Oberkochen, Germany). Images were converted to the tagged-information-file format and processed with the Adobe Photoshop program.

2.2.14 Miscellaneous techniques

To determine the concentration and purity of DNA solution, the OD at 260 nm and 280 nm was measured in a spectrophotometer. The absorption of 1 at OD_{260} is equivalent to 50 mg/ml of double stranded DNA. The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of the DNA with respect to contaminants that absorb in the UV, such as proteins. Molecular biology techniques were performed using standard protocols (Sambrook *et al*., 1989). Protein concentration was measured by the method of Bradford (Bradford, 1976). SDS-PAGE was performed by the method of Laemmli (Laemmli, 1970) in 10% vertical slab gels, unless otherwise stated. For immunoblot, proteins were transferred to nitrocellulose or PVDF membranes and immunostained with the indicated antibodies.

2.2.15 Statistical analysis and presentation of data

All experiments were performed at least thrice. In the case of western blot analysis representative data from one experiment are presented. All numerical data are expressed as mean ± SD. Data were analyzed using the two tailed t-test or ANOVA. Results were considered significant at the 5% level.

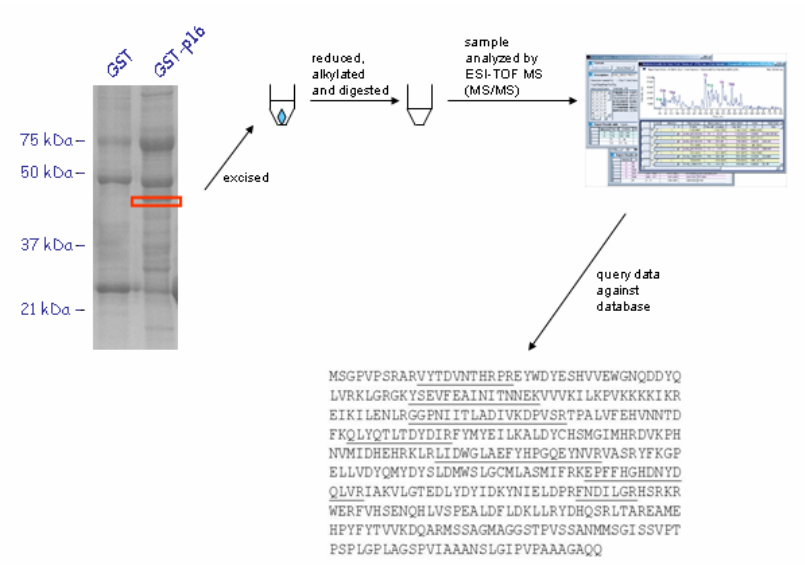

FIG. 1. Schematic diagram depicting the identification of various isolated p35-interacting proteins. From the gel, unique bands found in the sample lane, but not in the control, were excised. Proteins were reduced with DTT and alkylated with iodoacetamide, prior to digestion with modified sequencing grade trypsin. The digested samples were analyzed by mass spectrometry and sequences of some peptides were generated. Peptide sequences were queried against known databases to acquire their protein identities. Some here is the protein sequence of the catalytic α subunit of rat protein kinase CK2. Underlined are the partial sequences obtained from the mass spectrometry analysis.

Section 3

Results and Discussions

3.1 Isolation of p35-Associated Proteins and Identification of Protein Kinase CK2 as an Inhibitor of Neuronal Cdk5 kinase

3.2 Direct Regulation of Microtubule Dynamics by Protein Kinase CK2

Section 3 - Part I

3.1 Isolation of p35-Associated Proteins and Identification of Protein Kinase CK2 as an Inhibitor of Neuronal Cdk5 kinase

3.1.1 Introduction

Protein kinase CK2 is a serine/threonine protein kinase that is messenger-independent, ubiquitously expressed and highly conserved in evolution, indicating a vital cellular role for this kinase (Allende and Allende, 1995; Pinna and Meggio, 1997). CK2 phosphorylates a great number of substrates involved in essential processes, including cell growth and proliferation (Meggio and Pinna, 2003). Until recently, CK2 was believed to represent a kinase especially required for cell cycle progression in non-neural cells. At present, with respect to recent findings, few essential features suggest potentially important roles for this enzyme in specific neural functions. CK2 is very abundant in the brain, with numerous neural-specific substrates (Meggio and Pinna, 2003). In addition, some data have suggested that CK2 might play a role in processes underlying progressive disorders due Alzheimer's disease and ischemia.

Cdk5 plays important roles in the regulation of neuronal differentiation, degeneration and cytoskeletal dynamics (Dhavan and Tsai, 2001; Lee and Tsai, 2003). Monomeric Cdk5 has no enzymatic activity since its activation is dependent on its association with the neuronal-specific regulatory subunits p35 or p39 (Lew *et al*., 1994; Tsai *et al*., 1994; Tang *et al*., 1995). Cdk5 and p35 are essential for the proper development of the central nervous system since p35 and Cdk5 knockout mice display cell-positioning defects in the cerebral cortex (Chae *et al*., 1997; Ohshima *et al*., 1996). Cdk5 phosphorylates a number of cytoskeletal proteins and in turn mediates neurite outgrowth and neuronal migration during brain development (Baumann *et al*., 1993; Nikolic *et al*., 1998; Grant *et al*., 2001; Rashid *et al*., 2001; Ackerley *et al*., 2003; Xie *et al*., 2003b). There is growing evidence implicating aberrant regulation of

Cdk5 in neurodegeneration and cell death (Lim and Qi, 2003). Indeed p25, a truncated C-terminal fragment of p35, accumulates in AD brains and its associated Cdk5 kinase activity induces cytoskeletal disruption, morphological degeneration and apoptosis (Lee *et al*., 2000; Ahlijanian *et al*., 2000; Noble *et al*., 2003; Cruz *et al*., 2003).

Evidence has been accumulating in implicating Cdk5/p35 as a multifunctional enzyme that exists in many protein complexes in cells (Lim *et al*., 2003). In addition, biochemical studies of Cdk5 in bovine brain extract suggested the existence of inhibitory factors present together with Cdk5 and p35 in macromolecular protein complexes (Lee *et al*., 1996a). To learn more about the functional and regulatory roles of this kinase, we utilized recombinant p35 in affinity isolation of their binding proteins from rat brain homogenates and then identified using mass spectrometry. Herein, we present evidence showing that the catalytic α subunit of protein kinase CK2 as one of the component in the p35 protein complex. Both the catalytic subunit and the holoenzyme of CK2 are able to interact physically with p35. Using deletion mutants of p35, we show that CK2 binds to two separate regions located separately at the N- and C-terminal halves of p35. Intriguingly, the binding of CK2 to p35 prevents Cdk5 from associating with p35, thereby inhibiting the p35-mediated activation of Cdk5.

3.1.2 Results

3.1.2.1 Isolation of p35-binding proteins by affinity purification and co-immunoprecipitation

It has been shown that Cdk5/p35, but not Cdk5/p25, exists in macromolecular complexes in brain extracts, implying that the N-terminal 98 amino acids of p35 is important for interaction with other proteins (Lee *et al*., 1996a). Moreover, many proteins have been shown to interact with Cdk5 via their association with p35. Previous studies have reported p35 to be an unstable protein, and can be cleaved into a smaller p25 fragment in the presence of calcium-dependent proteases such as calpain (Lee *et al*., 2000). Hence, p35 was expressed as a GST-fusion protein with a C-terminus His_6-tag (GST-p35-His). Using sequential affinity purification, we were able to obtain a sufficiently pure form of the p35 protein. To test if the bacterial-purified recombinant proteins were functional, *in vitro* Cdk5 kinase assays were performed with these p35 and Cdk5 proteins and results obtained suggested that they are functional since the recombinant p35 proteins were able to activate Cdk5 activity (results not shown). To isolate proteins binding to p35, GST and GST-p35-His were utilized in affinity binding experiments with rat brain extracts. The bound proteins were either eluted at a high salt condition (1 M NaCl) or by boiling the GST/GST-p35-His immobilized beads in SDS-PAGE buffer. After resolving the eluted proteins by SDS-PAGE, several distinct polypeptide bands found in the GST-p35-His lane, but not in the GST control, were excised and subjected to in-gel digestion and mass spectrometry analysis. The identities of the isolated proteins were summarized in Table 1. Of the few proteins isolated, both NFM and actin have been previously reported to be p35-associated proteins (Grant *et al*., 2001; Humbert *et al*., 2000a).

Heat-shock protein 70 (HSP-70), β-tubulin, α-tubulin, drebrin A and neuronal protein 25 are novel putative p35-interacting proteins identified using this method.

In order to identify p35-interacting proteins that are found in complex with p35 in the cells, a co-immunoprecipitation approach was also employed. Antibody against p35 (C19) was incubated with pre-cleared rat brain extract and proteins were eluted by boiling in SDS-PAGE buffer. Proteins were resolved by SDS-PAGE and distinct polypeptide bands found in the C19 immunoprecipitate, but not in the IgG control, were excised for in-gel digest and mass spectrometry analysis. Table 1 summarized a list of proteins isolated using this method. This approach identified several proteins, including the catalytic subunit of phosphatidylinositol 3-kinase (PI3-K), dynamin I, pRb, c-Jun NH_2-terminus kinase 3 (JNK3), the catalytic subunit of calmodulin-dependent kinase 2 (CaMKIIα), CK1δ and actin that have been previously shown to be interacting proteins of Cdk5/p35 kinase (Li *et al*., 2003; Tan *et al*., 2003; Tomizawa *et al*., 2003; Lee *et al*., 1997; Li *et al*., 2002; Dhavan *et al*., 2002; Liu *et al*., 2001; Humbert *et al*., 2000a).

Table 1. Cdk5/p35-associated proteins identified. $^{\Psi}$ denotes our unpublished results.

Associated Protein	Putative function	Method of isolation
Heat-shock protein 70 (rodent)$^{\Psi}$	Chaperon protein	GST-p35-His_6 affinity isolation
β-tubulin $^{\Psi}$	Microtubule component	GST-p35-His_6 affinity isolation/ Co-immunoprecipitation
α-tubulin $^{\Psi}$	Microtubule component	GST-p35-His_6 affinity isolation
Neurofilament M (Grant *et al*., 2001)	Intermediate filament protein	GST-p35-His_6 affinity isolation
Drebrin A $^{\Psi}$	Actin-associated protein	GST-p35-His_6 affinity isolation
Neuronal Protein 25 $^{\Psi}$	Actin-binding protein	GST-p35-His_6 affinity isolation/ Co-immunoprecipitation
Actin (Humbert *et al*., 2000a)	Microfilament monomer	GST-p35-His_6 affinity isolation/ Co-immunoprecipitation
PI3-K, p110 catalytic subunit (Li *et al*., 2003)	Signaling intermediates in survival pathways	Co-immunoprecipitation
Dynamin I (Tan *et al*., 2003; Tomizawa *et al*., 2003)	Cdk5 substrate; Mediates vesicle fission in endocytosis	Co-immunoprecipitation
pRb (Lee *et al*., 1997)	Cdk5 substrate; cell cycle regulator; possibly mediates neuronal apoptosis	Co-immunoprecipitation
JNK-3 (Li *et al*., 2002)	Mediates neuronal apoptosis	Co-immunoprecipitation
CamKIIα (Dhavan *et al*., 2002)	Mediates synaptic plasticity, memory and learning	Co-immunoprecipitation
Casein kinase Iδ (Sharma *et al*., 1999)	Phosphorylates Cdk5 *in vitro*	Co-immunoprecipitation

3.1.2.2 Identification of CK2α as a p35-binding protein

As described above as well as in a previous report (Qu *et al*., 2002), we performed the isolation of p35-interacting proteins from rat brain extracts using an N-terminal (p16) fragment of p35, which comprises residues 1-149 of p35. It was produced as a GST-fusion protein and used as "bait" in the affinity "pull-downs". Bound proteins were eluted with high salt and subsequently resolved by SDS-PAGE. Several protein bands, inclusive of an approximately 45 kDa were clearly observed in GST-p16 pull-downs, but not in those from the GST control sample (Fig. 1A). To reveal its identity, this 45 kDa protein band was excised, subjected to in-gel digestion and mass spectrometry analysis. Sequence database searches with tandem mass spectrometric data generated from several prominent peptide signals showed that the sequences from these peptides matched those of rat CK2α, which is the catalytic subunit of protein kinase CK2 (Fig. 1A). To confirm this result, samples from a subsequent pull-down were used for immunoblot analysis. In addition to GST-p16, a shorter N-terminal fragment of p35, GST-p10 (a.a. 1-98 of p35), was also included in the pull-down analysis. As shown in Fig. 1B, a CK2α immunoblot clearly indicated that it was specifically pulled-down by GST-p16. Interestingly, GST-p10 failed to pull-down CK2α from the brain extract (Fig. 1B), implicating that the region spanning a.a. 99-149 of p35 is required for the association of CK2α.

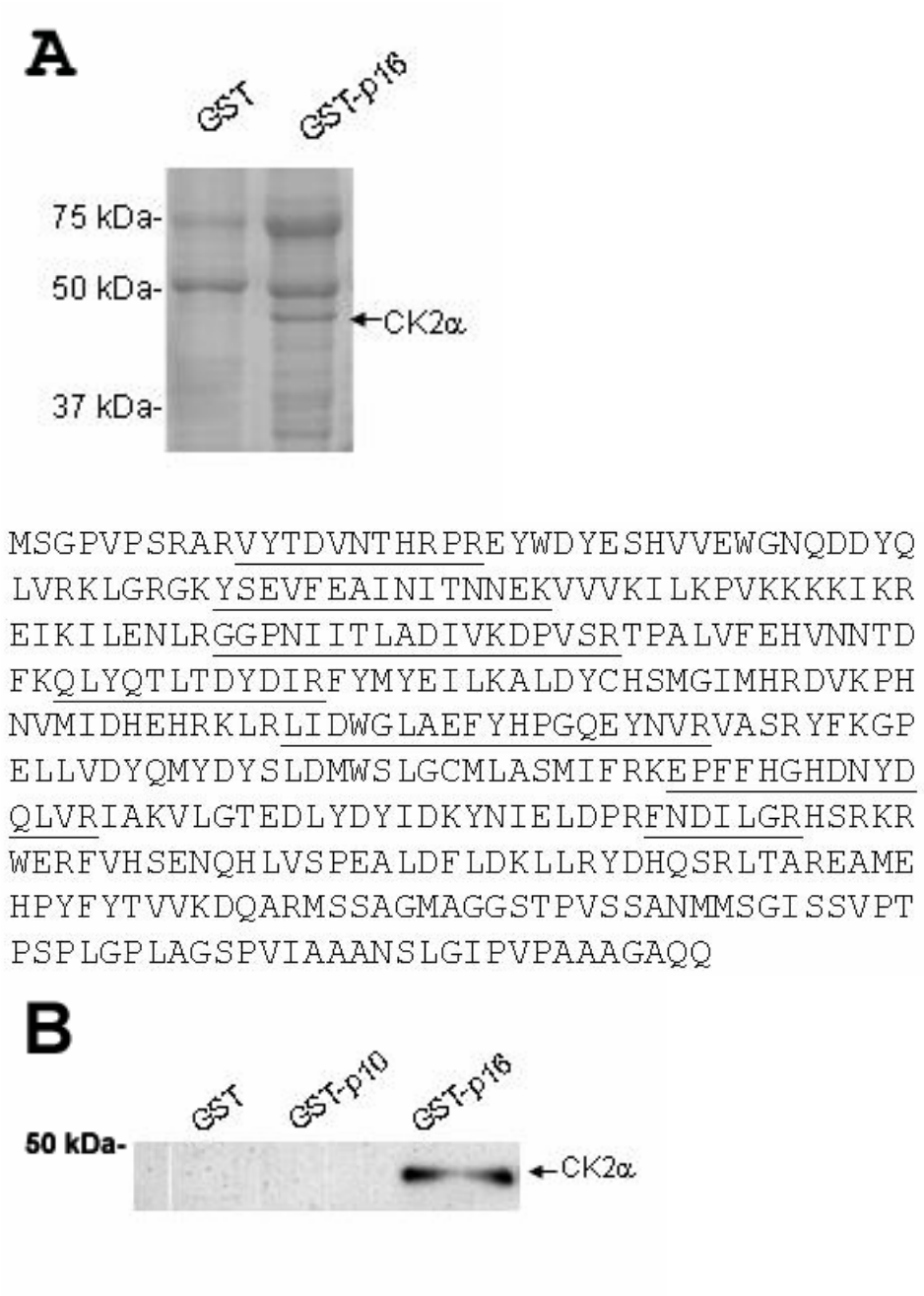

FIG. 1. Isolation of CK2α as a p35-binding protein from brain lysate. (A) GST and GST-p16 were used in the pull-down experiments of rat brain lysate as described in the "*Experimental Procedures*". Proteins co-precipitated through the GST-tagged proteins by GSH beads were separated by SDS-PAGE. The protein band at ~45 kDa in the GST-p16 lane was identified by mass spectrometry as the rat CK2α protein. Underlined are peptide sequences of rat CK2α identified by tandem mass spectrometry of the 45 kDa protein band. **(B)** Pull-downs of the rat brain lysate using GST, GST-p10 and GST-p16 were analyzed by anti-CK2α immunoblotting.

3.1.2.3 CK2α associates with p35 and Cdk5 in vivo

Cdk5 (with p35) is a multifunctional enzyme found in many macromolecular protein complexes (Lee *et al*., 1996a). To investigate the association of CK2 with p35 and Cdk5 *in vivo*, rat brain extracts were fractionated by size exclusion chromatography using a Superdex 200 column. Fractions were collected and analyzed by immunoblotting to examine elution patterns of Cdk5, p35 and the CK2 subunits. Figure 2A shows the elution profiles derived. Almost all p35 was found in the high molecular weight fractions, migrating nearly at the void volume of the column (Fig. 2A). Cdk5 was eluted across a fairly broad range of fractions (Fig. 2A), characteristic of various Cdk5 complexes present in brain extracts. Substantial amounts of Cdk5 were found in the high molecular weight fractions, co-fractionating with p35. The low molecular weight fractions corresponding to a molecular mass of ~30 kDa presumably represents the monomeric form of Cdk5. CK2α immunoreactivity was detected in the high molecular weight fractions of p35 and Cdk5 with the peak fractions closely aligned with that of p35 (Fig. 2A). Intriguingly, the high molecular weight fractions containing Cdk5, p35 and CK2α are completely devoid of CK2β (Fig. 2A), suggesting that CK2α but not the holoenzyme complexes with Cdk5 and p35 in the large protein complexes in brain. To further demonstrate the *in vivo* association of CK2α with Cdk5 and p35, proteins were immunoprecipitated from the rat brain lysate with antibodies directed to p35 and Cdk5. As shown in Figure 2B, CK2α was specifically detected in immunoprecipitates of both p35 and Cdk5. In addition, cellular localization patterns of CK2α and p35 were revealed by immunofluorescent staining of transfected COS-7 cells and NGF-induced PC-12 cells. Microscopic imaging of CK2α displayed a clearly defined co-localization with p35 in the cytoplasm (Fig. 2C), providing further evidence for the interaction between CK2α and Cdk5/p35.

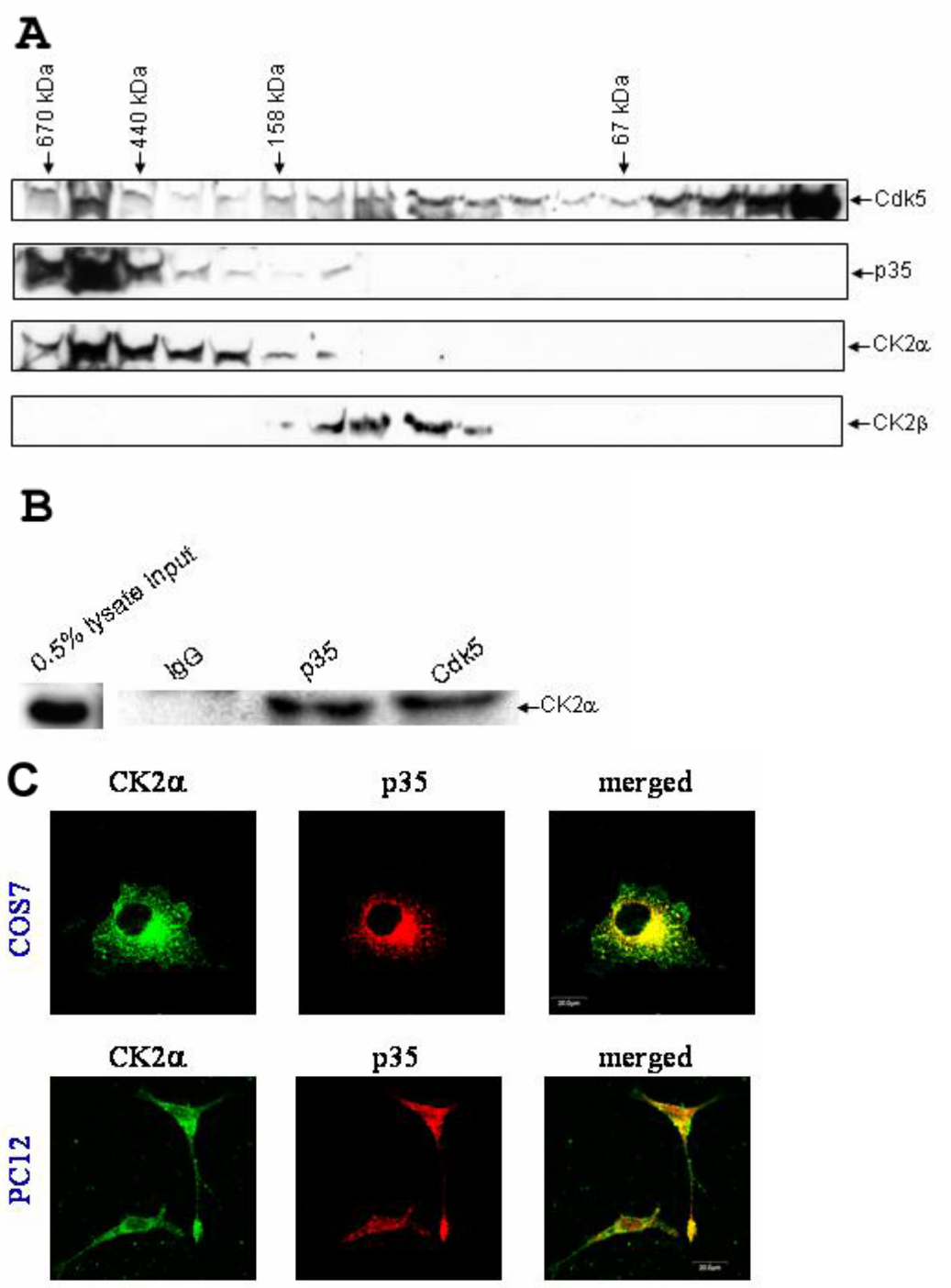

FIG. 2. *In vivo* association of CK2α with Cdk5 and p35. (A) Rat brain lysate containing 10 mg of proteins was analyzed by gel filtration chromatography using a Superdex 200 column as described under "*Experimental Procedures*". Proteins in alternate fractions were concentrated by acetone precipitation, resolved in 10% SDS-PAGE and subsequently immunoblotted with antibodies recognizing Cdk5, p35, CK2α and CK2β. **(B)** Rat brain lysate was subjected to immunoprecipitation with p35- and Cdk5-specific antibodies, followed by Western blotting with anti-CK2α antibody. Non-specific rabbit IgG was used in the immunoprecipitation as control. **(C)** *Top row*, COS-7 cells were co-transfected with the p35 and Myc-CK2α expression vectors. Immunostaining was performed using anti-p35 (red) and anti-Myc (green) antibodies on the transfected cells. *Bottom row*, double immunostaining was performed on NGF-induced PC-12 cells to label endogenous CK2α (green) and p35 (red) proteins. Bar = 20 μm

3.1.2.4 Direct association of CK2 with p35 and Cdk5

The physical association of CK2 with p35 and Cdk5 was further examined using purified recombinant proteins. The CK2 holoenzyme comprises two α and two β subunits (Niefind *et al*., 2001). GST-tagged p35 and Cdk5 were individually incubated with CK2α, CK2β or the holoenzyme, respectively. The GST-tagged proteins were immobilized on GSH affinity beads and co-precipitated proteins were analyzed by immunoblotting using a mixture of CK2α and CK2β antibodies. Western blots clearly showed an interaction between p35 and either CK2α or the holoenzyme but not CK2β (Fig. 3A). In addition to p35, Cdk5 also displayed direct binding to CK2α although it failed to interact with the CK2 holoenzyme (Fig. 3A). These results indicated that p35 is able to associate with CK2 holoenzyme via its interaction with CK2α, while Cdk5 binds only to CK2α.

Upon identifying p35 as a binding partner of CK2, we proceeded to delineate the CK2-binding regions in p35. As CK2α was isolated by the p16 fragment but not the p10 fragment of p35, two truncated fragments of p16, GST-p35 (a.a. 1-141) and GST-p35 (a.a. 53-149), were constructed and the respective recombinant proteins with a GST tag were prepared. Two C-terminal constructs of p35, namely p25 and p35 (a.a. 150-307), were also employed side-by-side in the GST pull-down assays. As shown in figure 3B, p16, but none of the shorter constructs, could interact with CK2α, suggesting that any further truncation of p16 would destroy its CK2-binding capability. Unexpectedly, p25 and p35 (a.a. 150-307) also exhibited interaction with CK2α although the bindings were relatively weak (Fig. 3B). The C-terminal half of p35 constitutes the Cdk5-binding and activating domain, which spans a.a. 150-292

(Tang *et al*., 1997; Poon *et al*., 1997). Thus, CK2 appears to interact with two regions of p35, one of which overlaps with the Cdk5-binding domain.

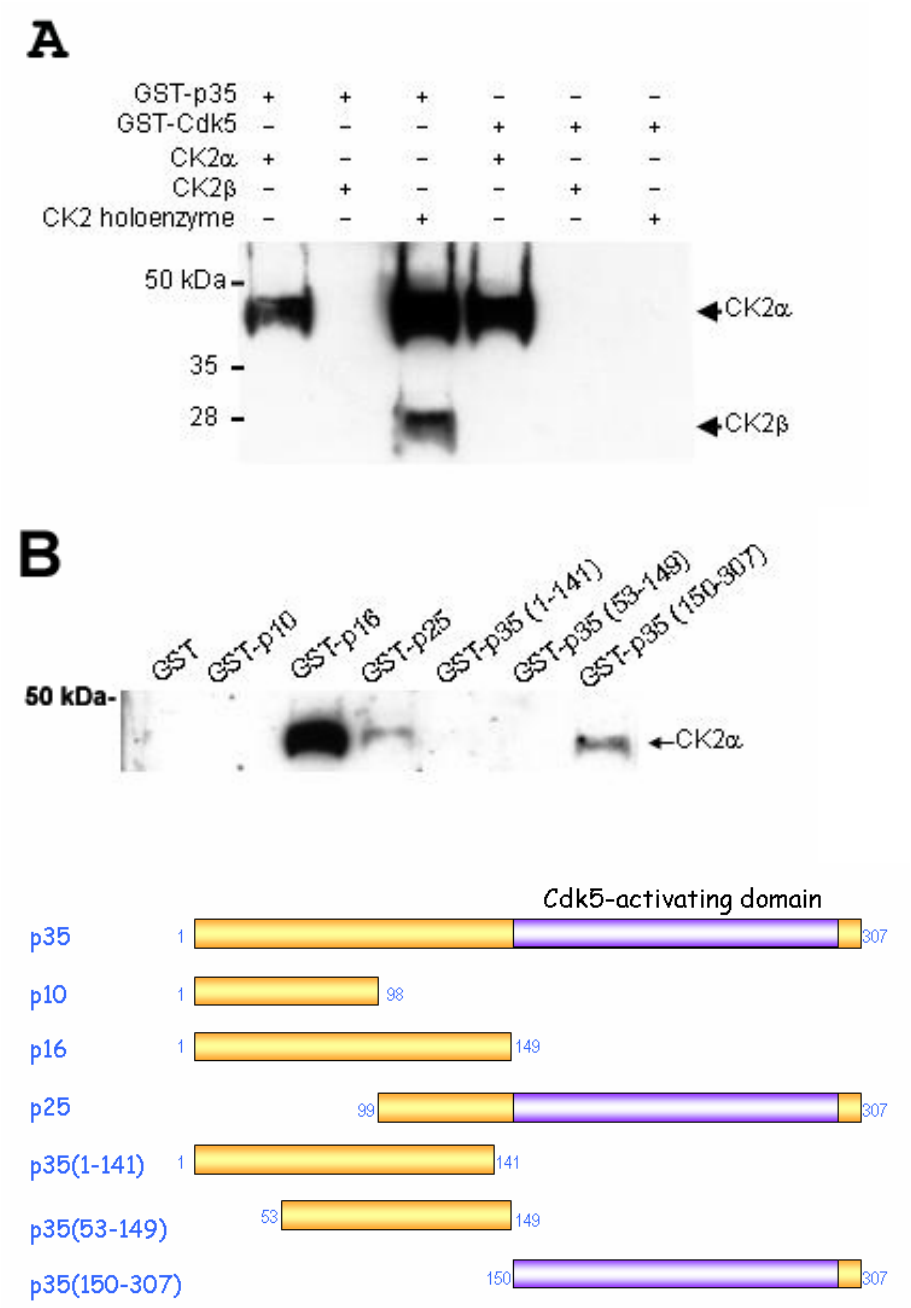

FIG. 3. Direct binding of CK2 to p35 and Cdk5. (A) CK2 associates with p35 and Cdk5. 10 µg of GST-p35 or GST-Cdk5 were incubated individually with CK2α (0.5 µg), CK2β (0.5 µg) and the CK2 holoenzyme (1 µg). After precipitating the GST proteins using GSH beads, bound proteins were immunoblotted using a mixture of CK2α and CK2β antibodies. **(B)** Mapping CK2-binding domain in p35. 10 µg of GST-tagged fragments of p35 or GST were incubated with CK2α (0.5 µg). The GST proteins were then retrieved by GSH beads and analyzed by anti-CK2α immunoblotting. A schematic diagram depicting the various deletion constructs of p35 is shown.

3.1.2.5 CK2 inhibits Cdk5 activation

It has been established that Cdk5 can be activated *in vitro* by reconstitution with p35 or its truncated form p25 (Qi *et al*., 1995). To understand the function of such interactions between CK2 with p35 and Cdk5, we carried out *in vitro* Cdk5 kinase assays using recombinant proteins to examine the potential effects of CK2. Cdk5 kinase assay shows that reconstitution of Cdk5 and p35 in the presence of CK2α or CK2 holoenzyme resulted in an inhibition of the Cdk5 kinase activity in a dose-dependent manner (Fig. 4A). Compared to CK2α, the holoenzyme of CK2 exhibited a slightly higher potency in inhibiting Cdk5 activity. Since p25 constitutes a second binding site for CK2, we went on to examine the effect of CK2 on the activation of Cdk5 by p25. Similar to the p35 results, CK2α and CK2 holoenzyme strongly inhibited Cdk5 activity when they were included in the reconstitution mixtures of Cdk5 and p25 (Fig. 4B). Thus, CK2 inhibited Cdk5 activation by p35 and p25 with similar potencies.

Cdk5 is a ubiquitously expressed protein. To further investigate the mode of Cdk5 inhibition by CK2, we introduced p35 and CK2 into cultured COS-7 cells by transient transfection. p35 was immunoprecipitated to test its associated Cdk5 activity. When p35 was co-transfected with an empty vector, a high Cdk5 kinase activity was obtained from the p35 immunoprecipitates (Fig. 5A). However, when p35 was transfected with CK2α or the CK2 holoenzyme expression constructs, the p35-associated Cdk5 activities were significantly reduced (Fig. 5A). Western blots detected the over-expressed CK2α and CK2 holoenzyme in the immunoprecipitates of p35, corroborating the association of p35 and CK2. In agreement with the *in vitro*

reconstitution assays, the expression of the CK2 holoenzyme (CK2α/CK2β) imposed a stronger inhibition on the p35-associated Cdk5 activity compared to the CK2α.

To evaluate the physiological role of CK2 on Cdk5 activity, we proceeded to knock down CK2α/α' in human neuroblastoma SH-SY5Y cells using a siRNA duplex. As shown by cell lysate immunoblots, the introduction of CK2α/α'-specific siRNA led to a significant decrease in the cellular CK2α protein level (Fig. 5B), while the protein contents of Cdk5 and p35 were not affected. To assess the knockdown effect on Cdk5 activity, p35 was immunoprecipitated to determine its associated Cdk5 activity. The introduction of CK2α/α'-specific siRNA yielded a notably higher Cdk5 activity as compared to the control sample (scrambled siRNA sequence) (Fig. 5C), implicating the Cdk5 inhibitory effect of CK2 in the cells.

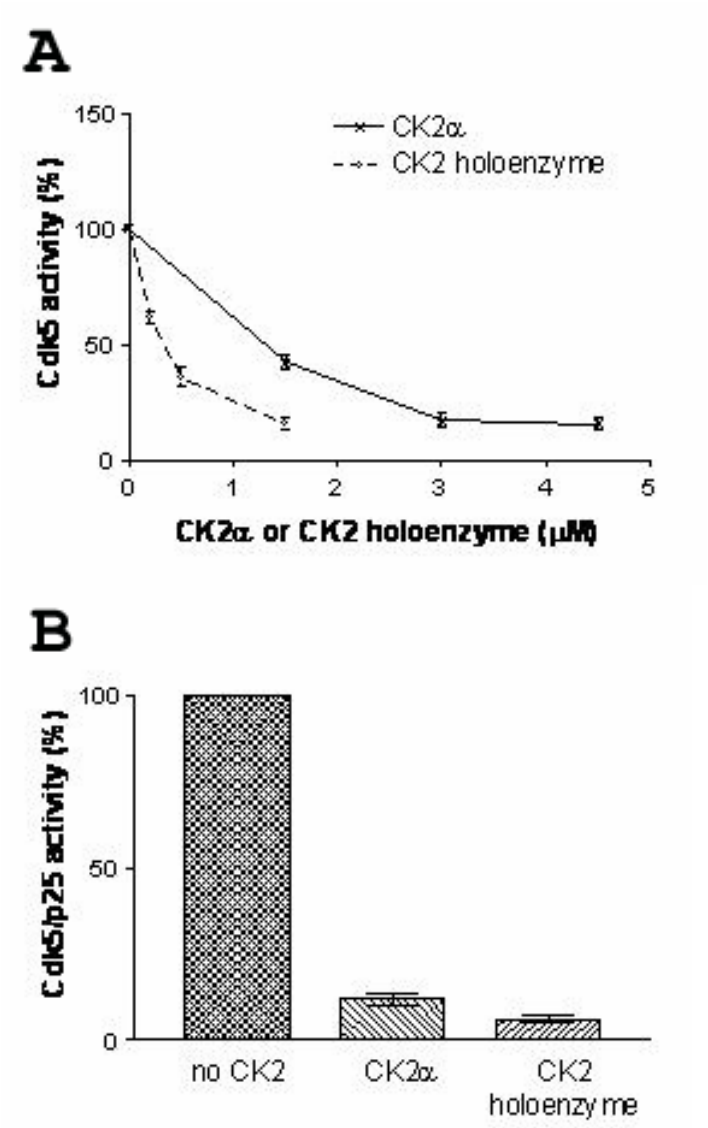

FIG. 4. CK2 inhibits Cdk5 activation *in vitro*. (A) CK2 inhibits the p35 activation of Cdk5. GST-Cdk5 (0.5 μg) was mixed and incubated with GST-p35 (0.5 μg) in the absence and presence of increasing amounts of CK2α or CK2 holoenzyme. The samples were incubated for 1 hr at room temperature before being assayed for the Cdk5 kinase activity. **(B)** CK2 inhibits the p25 activation of Cdk5. GST-Cdk5 (0.5 μg) was incubated with GST-p25 (0.5 μg) and CK2α (5 μg) or CK2 holoenzyme (5 μg) for 1 hr at room temperature. The Cdk5 kinase activity was then determined and expressed as percentages of the Cdk5 activity in the absence of the CK2 proteins. Data shown here are averages of three separate experiments.

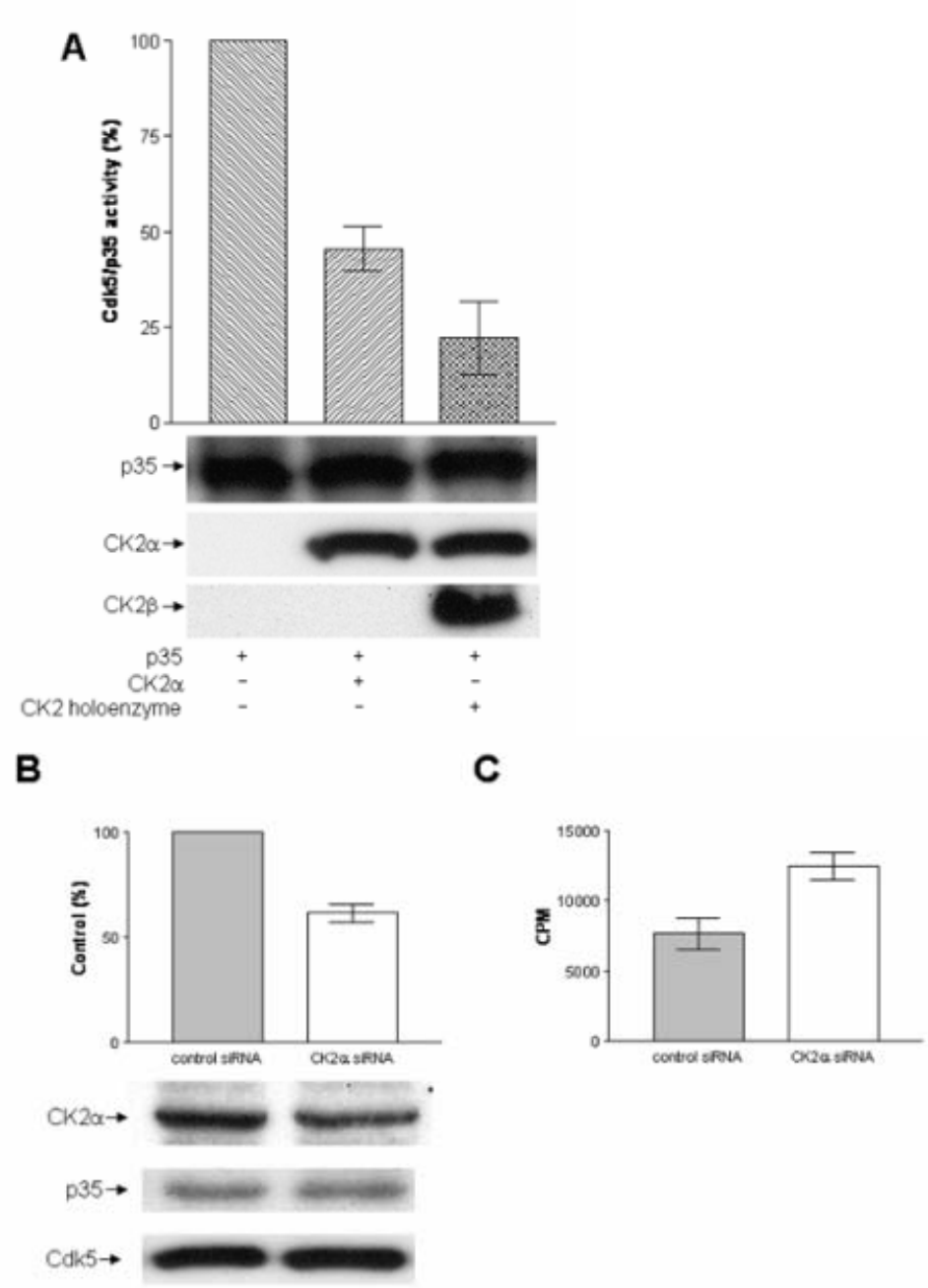

FIG. 5. (A) Cdk5 is inhibited by CK2 in transfected cells. The p35 expression construct was co-transfected with the plasmid expressing CK2α or the holoenzyme CK2α/CK2β (CK2 holoenzyme) into COS-7 cells. In a control experiment, p35 was co-transfected with the empty vector of the CK2 constructs. The expressed proteins of CK2α and CK2β contain V5 and Myc tags, respectively. Following immunoprecipitation of p35, the activity of co-precipitated Cdk5 was determined and expressed as percentages of that in the control sample. The plot represents an average of three individual experiments. The anti-p35 immunoprecipitates were subjected to immunoblotting to visualize p35, CK2α and CK2β by antibodies against p35, V5 and Myc, respectively. **(B-C) CK2 inhibits Cdk5 activity in neuroblastoma cells.** SH-SY5Y cells were introduced with the siRNA of human CK2α/α' or a scrambled sequence. **(B)** The knockdown of CK2α/α' was monitored by anti-CK2α, p35 and Cdk5 immunoblotting. The protein level of CK2α in the knockdown cells was expressed as a percentage of that in the control sample. **(C)** Following immunoprecipitation of p35, the activity of co-precipitated Cdk5 was determined. Data shown in the graph are the averages of three individual experiments ($\rho = 0.005$).

3.1.2.6 CK2 inhibits Cdk5 in a phosphorylation-independent manner

CK2 is a protein Ser/Thr kinase that phosphorylates a multitude of proteins (Meggio and Pinna, 2003). To investigate the possible role of CK2 kinase activity in the inhibition of Cdk5, recombinant CK2αK68A, a kinase-dead mutant of CK2α, was employed. When recombinant CK2αK68A was added into the reconstitution mixture with Cdk5 and p35, the Cdk5 activity was strongly inhibited. CK2αK68A displayed an inhibitory activity that is similar to the wild-type protein on Cdk5 activity (Fig. 6). Cdk5 inhibition was also achieved using the kinase-dead holoenzyme of CK2, which was reconstituted from CK2αK68A and CK2β (Fig. 6). From these results, we concluded that the CK2 inhibition of Cdk5 is independent of its kinase activity.

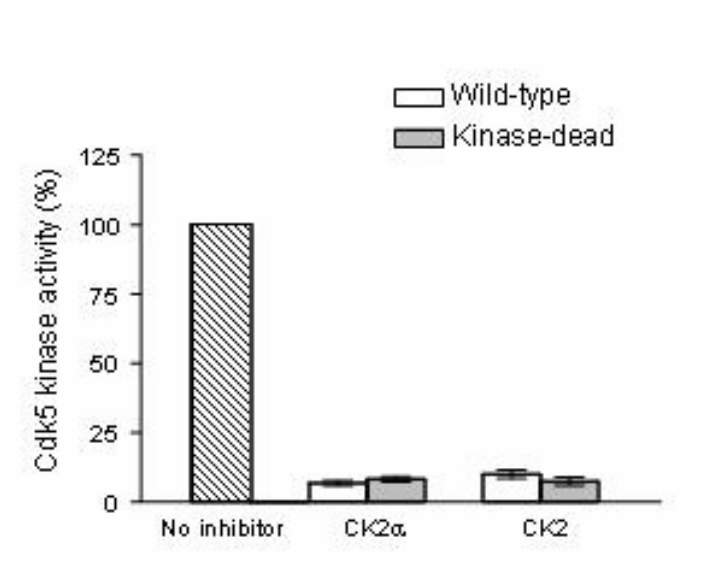

FIG. 6. The kinase activity of CK2 is not required for the inhibition of Cdk5. GST-Cdk5 (0.5 μg) was mixed with GST-p35 (0.5 μg) and one of the following proteins (5 μg) for each sample: CK2α, CK2αK68A, the wild-type holoenzyme CK2α/CK2β and the kinase-dead holoenzyme CK2αK68A/CK2β. The samples were incubated for 1 hr at room temperature before the Cdk5 kinase assay. Data shown in the graph represent an average of three separate experiments.

3.1.2.7 CK2 blocks complex formation between Cdk5 and p35

To further characterize the inhibition of Cdk5 activity by CK2, we designed kinase assay experiments where Cdk5, p35 and the CK2 proteins were pre-reconstituted in different orders. Interestingly, it was found that the Cdk5 inhibition depends on the order of the protein reconstitution. The Cdk5 activity was strongly blocked if Cdk5, p35 and CK2 were added in the following two ways: p35 was incubated with CK2 prior to the addition of Cdk5; or Cdk5, p35 and CK2 were added simultaneously during the incubation (Fig. 7). However, if Cdk5 and p35 were incubated prior to the addition of CK2, the preformed complex of Cdk5-p35 was completely resistant to CK2 inhibition (Fig. 7). Thus, CK2 acts to block the Cdk5 activation, but does not affect the activity of Cdk5 already in complex with p35.

In order to understand how CK2 inhibits Cdk5 activation, we utilized pull-down binding assays to examine if CK2 competes with Cdk5 in binding to p35. GST-p35 was pre-incubated with or without an excessive amount of CK2 (CK2α or CK2 holoenzyme). After GST-p35 was retrieved using GSH-beads and unbound materials were washed off, it was subjected to a binding assay with Cdk5. As shown in Figs 8A/B, p35 preincubated with CK2 completely lost its ability to interact with Cdk5. We extended this analysis to test whether CK2 can bind to the complex of Cdk5-p35. This time, GST-p35 was pre-complexed with Cdk5 and subsequently subjected to CK2 binding. As shown in Figs. 8C & 8D, the complex of Cdk5-p35 had no observed binding activity to CK2. Hence, CK2 and Cdk5 are mutually exclusive in their binding to p35. However, they are not able to displace one another if either protein is pre-complexed to p35.

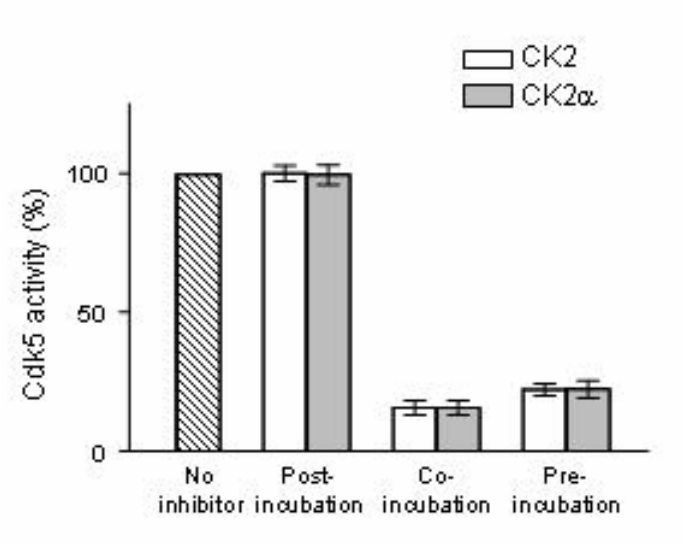

FIG. 7. **CK2 blocks Cdk5 activation but does not affect the activity of pre-activated Cdk5.** 5 μg of CK2 or CK2α were incubated with GST-p35 for 1 hr, followed by addition of GST-Cdk5 (1 μg) and a further incubation for 1 hr (pre-incubation) at room temperature. For co-incubation, all three proteins were mixed together at the same time. For post-incubation, GST-p35 and GST-Cdk5 were mixed and incubated for 1 hr before the addition of CK2 or CK2α and a further incubation for 1 hr. In the "No inhibitor" sample, GST-Cdk5 and GST-p35 were reconstituted without the CK2 proteins for 2 hrs. The Cdk5 kinase activity in each sample was then assayed. Data shown here are representatives of three separate experiments and expressed as percentages of the Cdk5 activity of the "No inhibitor" sample.

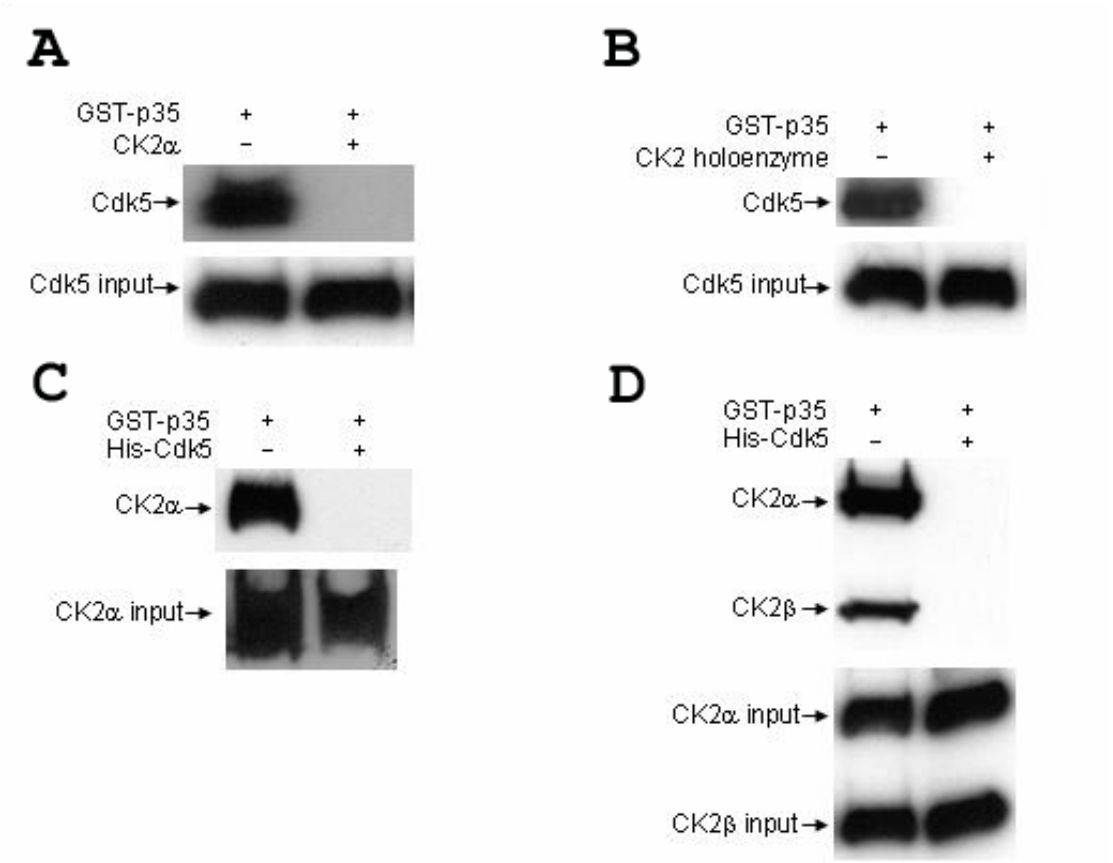

FIG. 8. CK2 and Cdk5 are mutually exclusive in their binding to p35. (**A**) GST-p35 (1 μg) was pre-incubated with or without CK2α (5 μg) for 1 hr at room temperature. GST-p35 was retrieved using GSH beads to remove unbound materials, and then subjected to incubation with His-Cdk5 (8 μg) for 1 hr at room temperature. After the incubation, His-Cdk5 bound to the beads was analyzed by Cdk5 immunoblotting. An immunoblot of the His-Cdk5 input in the incubation was shown in the bottom row. (**B**) The competitive binding assay described in (*A*) was performed using the holoenzyme CK2α/CK2β instead of CK2α. (**C**) GST-p35 (1 μg) was pre-incubated with or without His-Cdk5 (5 μg) for 1 hr at room temperature. After GST-p35 was retrieved using GSH beads and unbound materials were removed by washing, the beads were incubated with CK2α (8 μg) for 1 hr at room temperature. CK2α bound to p35 immobilized to the GSH beads was detected by Western blotting. An immunoblot of the CK2α input in the incubation was shown in the bottom row. (**D**) The competitive binding assay described in (*C*) was performed using the holoenzyme CK2α/CK2β in place of CK2α.

3.1.3 Discussion

Using both affinity isolation as well as co-immunoprecipitation approaches, a number of Cdk5/p35-interacting proteins were isolated (Table 1). Several of these proteins are known to participate in controlling cytoskeletal organization while a handful of others are proteins involved in cellular signal transduction processes. Among the cytoskeletal proteins identified, drebrin is an actin-associated protein where it co-localizes with actin filaments and has been suggested to regulate assembly and disassembly of actin filaments (Shirao *et al*., 1994; Asada *et al*., 1994). p39 has been previously reported to bind actin. Hence, their interaction with p35 suggests that Cdk5 may have a role in modulating cellular actin dynamics. Microtubule is a major component of the cytoskeleton. The interaction between p35 and α- and β-tubulin imply a role for p35 in the regulation of cellular microtubule dynamics.

Several proteins with putative signaling functions were also isolated and one of them is the catalytic subunit of protein kinase CK2. CK2 is a serine/threonine kinase highly conserved in eukaryotic cells. It is a ubiquitously expressed pleiotropic enzyme that is involved in the control of various cellular processes such as cell cycle, apoptosis, transcriptional regulation and signal transduction (Allende and Allende, 1995; Pinna and Meggio, 1997; Litchfield, 2003; Meggio and Pinna, 2003). CK2 is much more abundant in brain than any other tissue. There appear to be a myriad of CK2 substrates in neural cells which have clear implications in neural development, neuritogenesis, synaptic transmission and plasticity (Blanquet, 2000). The holoenzyme of CK2 is generally a heterotetramer, composed of two catalytic α or α' and two regulatory β subunits (Niefind *et al*., 2001). The α and α' subunits are catalytically active, whereas the β-subunit is inactive but appears to confer stability to

the holoenzyme and to modulate the enzyme activity and substrate specificity by targeting the holoenzyme to its substrates (Allende and Allende, 1995; Pinna and Meggio, 1997). In addition, several lines of evidence have indicated that CK2 subunits can exist individually *in vivo* to interact with other cellular proteins, implying that the CK2 subunits may have biological functions other than those assigned to the holoenzyme (Chen *et al*., 1997; Chen and Cooper, 1997; Heriche *et al*., 1997; Guerra *et al*., 1999; Litchfield, 2003).

Evidence presented here identifies the catalytic α subunit of CK2 as an interacting partner of p35 and Cdk5. Based on the protein co-elution profiles of the rat brain lysate, CK2α but not the CK2 holoenzyme coexists with p35 and Cdk5 *in vivo*, although the holoenzyme displayed p35-binding capacity *in vitro*. Interestingly, the elution patterns of α- and β-subunits of CK2 were quite different, although they co-eluted in certain fractions. Increasing data had suggested that there are free populations of both CK2 subunits, either totally on their own or in association with cellular structures (Guerra and Issinger, 1999). Moreover, CK2β is associated with the plasma membrane (Faust and Montenarh, 2000) and the rat brain lysate is devoid of membrane-associated protein (detergent-free fraction), these would account for the distinct elution profiles between the subunits of CK2. In agreement with these results, CK2α was observed in p35- as well as Cdk5-immunopecipitates from the rat brain lysate. In respect to these observations, further characterization showed that CK2 and Cdk5 interact with p35 in a mutually exclusive manner. *In vivo*, it is possible that there are independent pools of CK2 proteins that are associated with p35, as well as presence of p35-associated Cdk5 kinase. Hence, the binding of CK2 to p35 prevents Cdk5 from associating with p35 and thereby inhibiting the p35-associated Cdk5

activation. In agreement with the results from the *in vitro* inhibition assays, the co-expression of CK2 and p35 in COS-7 cells inhibited the Cdk5 activation. In addition, the knockdown of CK2α/α' significantly enhanced the p35-associated Cdk5 activity in the neuroblastoma cells. Although the Cdk5-inhibitory mechanism of CK2 is distinct from the Kip/Cip family of Cdk inhibitors (CKIs), it is reminiscent of the Cdk4/6 inhibition by the INK4 family members, which interact with both Cdk4/6 and cyclin D to block the Cdk4/6 association with cyclin D (Russo *et al*., 1998; Guan *et al*., 1996; Hirai *et al*., 1995). Taken together, these support the notion that p35, Cdk5 and the CK2 proteins may form independent protein complexes in the cells.

CK2 was found to bind p35 in two regions that are separately located in the N- and C-terminal halves. Given the results that CK2 displayed an inhibitory activity towards Cdk5 activation by p25 and p25 (which retains one of the two CK2-binding sites that lies within the Cdk5-binding domain at the C-terminal half of p35), it is tempting to conclude that CK2 competes with Cdk5 in binding to p35 at the Cdk5-binding domain, and thereby prevents the association of p35 with Cdk5. Perhaps, the binding of CK2 to the N-terminal region of p35 prevent access to Cdk5. The binary complexes of p35-CK2 or p35-Cdk5 appear to be stable and cannot be dissociated by excessive amounts of Cdk5 or CK2, respectively. CK2 may act as a sentinel molecule in cells to control Cdk5 activation. Upon dissociation of CK2 from p35, p35 binds to and activates Cdk5, which in turn catalyses the phosphorylation of p35, promoting p35 degradation via the proteasome-dependent pathway (Patrick *et al*., 1998). Conceivably, the CK2 association prevents unwanted Cdk5 activation and p35 turnover, thereby preserves p35 for prompt Cdk5 activation. Thus, it would be intriguing to understand how p35 is released from its association with CK2. In

addition, the p35-CK2 interaction might have some effect on CK2 activity, which may allow us to further understand the regulation of CK2 cellular functions.

CK2 has a broad substrate spectrum which includes Cdc2 (Russo *et al*., 1992). We have examined if the kinase activity of CK2 is involved in its inhibition of Cdk5. Using the kinase-dead mutant of CK2, we found that the Cdk5-inhibiting activity of CK2 is independent of its kinase activity. This was corroborated with the results from the competitive protein binding assays. It becomes clear that CK2 imparts a direct inhibition on Cdk5 simply by competing with Cdk5 for the binding to p35, but not through its kinase activity. As a multifunctional enzyme, CK2 is thought to execute its functions through its phosphorylation of a myriad of proteins. The results presented here revealed a new CK2 function that is not linked to, or caused by its intrinsic kinase property.

In conclusion, this study has presented the identification and characterization of a novel inhibitor of Cdk5. To date, all identified functions of Cdk5 are inextricably linked to the phosphorylation of its substrates. Our results revealed a novel regulatory mechanism of Cdk5 activation and possibly its actions in brain. The aberrant activation of Cdk5 by p25, a proteolytic product of p35, occurs during neurodegeneration and abnormally high activity of Cdk5 is detrimental to cell survival (Nguyen *et al*., 2001; Patrick *et al*., 1999). Since CK2 exhibited strong inhibition of Cdk5 activation by p25, the loss of this inhibitory mechanism might be one of the deregulated mechanisms during the neuronal degeneration. On the other hand, it has been proposed that CK2 is involved in processes underlying cell survival and has anti-apoptotic functions (Pinna, 2002; Ahmed *et al*., 2002; Litchfield, 2003).

CK2 may conceivably act to protect the cells against neurotoxicity through its inhibitory effect on Cdk5. Thus, the function described here for CK2 also provides a mechanistic possibility of how CK2 may support cell viability in the nervous system.

Section 3 - Part II

3.2 Direct Regulation of Microtubule Dynamics by Protein Kinase CK2

3.2.1 Introduction

Microtubules are fibrous elements in the cytoplasm of eukaryotic cells where they perform a wide variety of functions. Microtubules are major organizers of the cell interior and are vitally involved in motility events such as chromosome migration during cell division. To fulfill their physiological function, microtubule arrays have to undergo dramatic changes in their spatial arrangements and this depends on, to a large extent, the complex and special dynamic properties of the individual polymers. A few lines of evidence have implicated CK2 in the regulation of microtubule cytoskeleton reorganization (Diaz-Nido *et al*., 1988; Serrano *et al*., 1987; Serrano *et al*., 1989). CK2 was localized to microtubular structures such as the mitotic spindle of dividing cells and was found to associate with a cold-stable fraction of microtubules from the rat brain (Diaz-Nido and Avila, 1992; Serrano *et al*., 1989). More recently, the α and α' subunits were shown to bind tubulin in a Far-Western assay (Faust *et al*., 1999). Further, CK2 is able to phosphorylate a number of proteins associated with microtubule, including MAP1B and a neuron-specific β-tubulin isotype (Meggio and Pinna, 2003). The phosphorylation of MAP1B was proposed to facilitate its microtubule association and thereby microtubule assembly, while the physiological role of the neuronal β-tubulin isotype phosphorylation is still unclear (Diaz-Nido *et al*., 1988; Diaz-Nido *et al*., 1990). Despite these findings, a direct role for CK2 in regulating microtubule stability has not yet been established.

In the present study, we have investigated the physical association of CK2 with microtubules and documented a direct effect of CK2 on microtubule dynamics. Our results show that CK2 is a microtubule-associated protein (MAP) that induces

microtubule assembly and bundling *in vitro*. The CK2-polymerized microtubules appear to be stable against cold treatment. In cultured cells, knockdown of CK2α/α' has a severe effect on the microtubule stability, indicating that CK2 is important for microtubule integrity *in vivo*. A kinase-inactive mutant of CK2 displayed similar microtubule-polymerizing and stabilizing activity *in vitro* and *in vivo*. Thus, the microtubule-assembling and stabilizing action of CK2 is independent of its kinase activity.

3.2.2 RESULTS

3.2.2.1 CK2 forms a direct complex with microtubules

The possible association between CK2 and microtubules was probed by a series of binding assays using recombinant CK2 and purified MAP-free tubulin as well as pre-assembled microtubules. Both the α- and β-tubulin was found to associate with the catalytic α subunit as well as the holoenzyme of CK2 (Fig. 1A). CK2β alone did not bind tubulin (Fig. 1B), which is in agreement with a previous observation made using Far-Western blotting (Faust *et al*., 1999). To verify the microtubule association of CK2, taxol-assembled microtubules were incubated with the CK2 holoenzyme or its individual subunits. The microtubules were then pelleted by centrifugation to test whether these proteins were co-precipitated with the microtubules. Consistently, CK2α and the holoenzyme of CK2 were found to associate with the microtubule pellets, whereas CK2β and GST (as a control) failed to co-precipitate with the microtubules (Fig. 1C). These results indicate that the CK2 holoenzyme associates with microtubules through CK2α.

Cellular localization of CK2 to microtubule networks was revealed by immunofluorescent staining of cultured COS-7 cells. Microscopic imaging of the endogenous CK2α and CK2β revealed its colocalization with the microtubule network, particularly at the cell periphery (Fig. 2A). In addition, pools of tubulin existed as free heterodimers or polymers (microtubules) were differentially extracted from the cultured cells and the extent of codistribution of CK2 with these fractions was examined (Lieuvin *et al*., 1994). Both CK2α and CK2β appeared in the microtubule fraction as well as the fraction of free tubulin heterodimers, though there appeared to be more CK2β in the microtubule fraction (Fig. 2B). Taken together with the results from

the *in vitro* binding assays, this data provide evidence for direct association of CK2 with cellular microtubules.

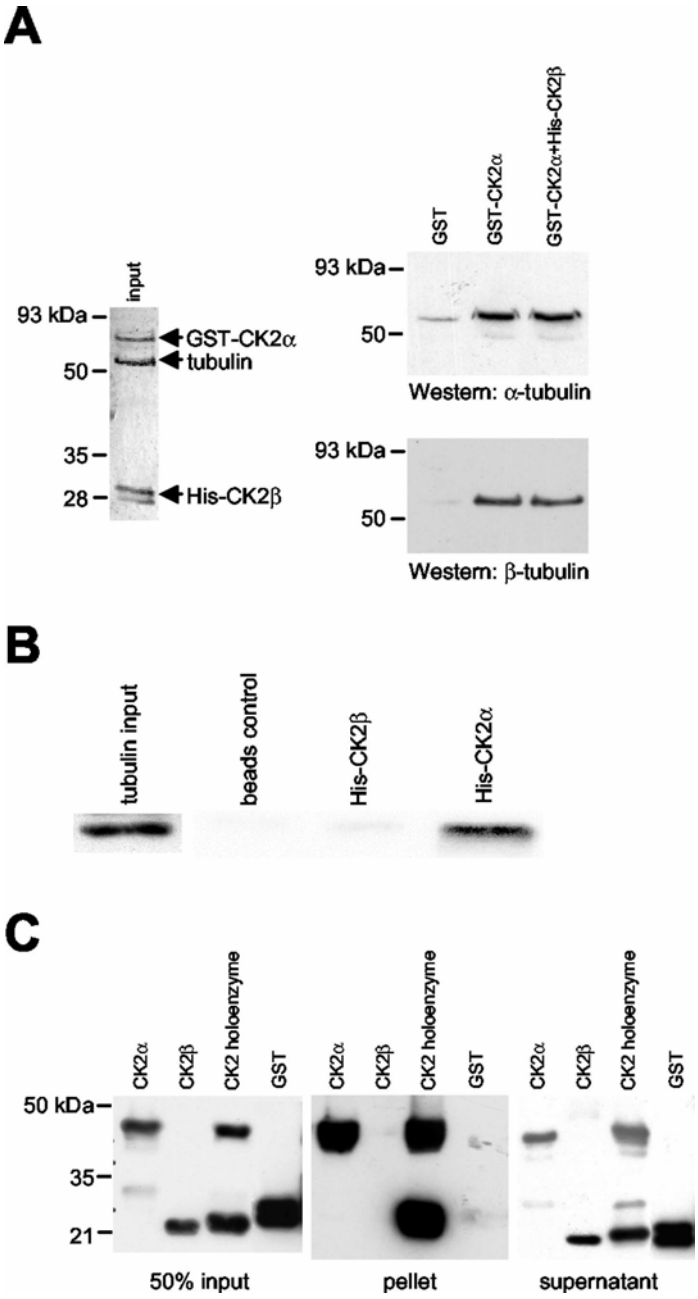

FIG. 1. Microtubule association of CK2. (A) Direct interaction of CK2 and tubulin heterodimers. 5 μg of GST, GST-CK2α or a complex of GST-CK2α/His-CK2β were incubated with 2.5 μg of purified tubulin. The GST fusion proteins were then retrieved using GSH beads and bound proteins were analyzed by immunoblotting with antibodies recognizing α- and β-tubulin. The protein input column was visualized by Coomassie Blue staining. **(B)** CK2β does not interact physically with tubulin. 5 μg of His-CK2α or His-CK2β, were incubated with 2.5 μg of purified tubulin. There was no His-CK2α or His-CK2β in the beads control sample. After pull-down with Ni-NTA beads, bound proteins were analyzed by anti-β-tubulin immunoblotting. **(C)** Direct association of CK2 with microtubules. 1 μg of GST, CK2α, CK2β or the CK2 holoenzyme was incubated with microtubules pre-assembled using taxol from 10 μg of purified tubulin. After precipitation of the microtubules, proteins in the supernatant and the microtubule pellet were analysed by immunoblotting using an antibody mixture recognizing GST, CK2α and CK2β. The protein input (50%) shows the amount of the individual proteins used in this experiment, as analyzed by immunoblotting.

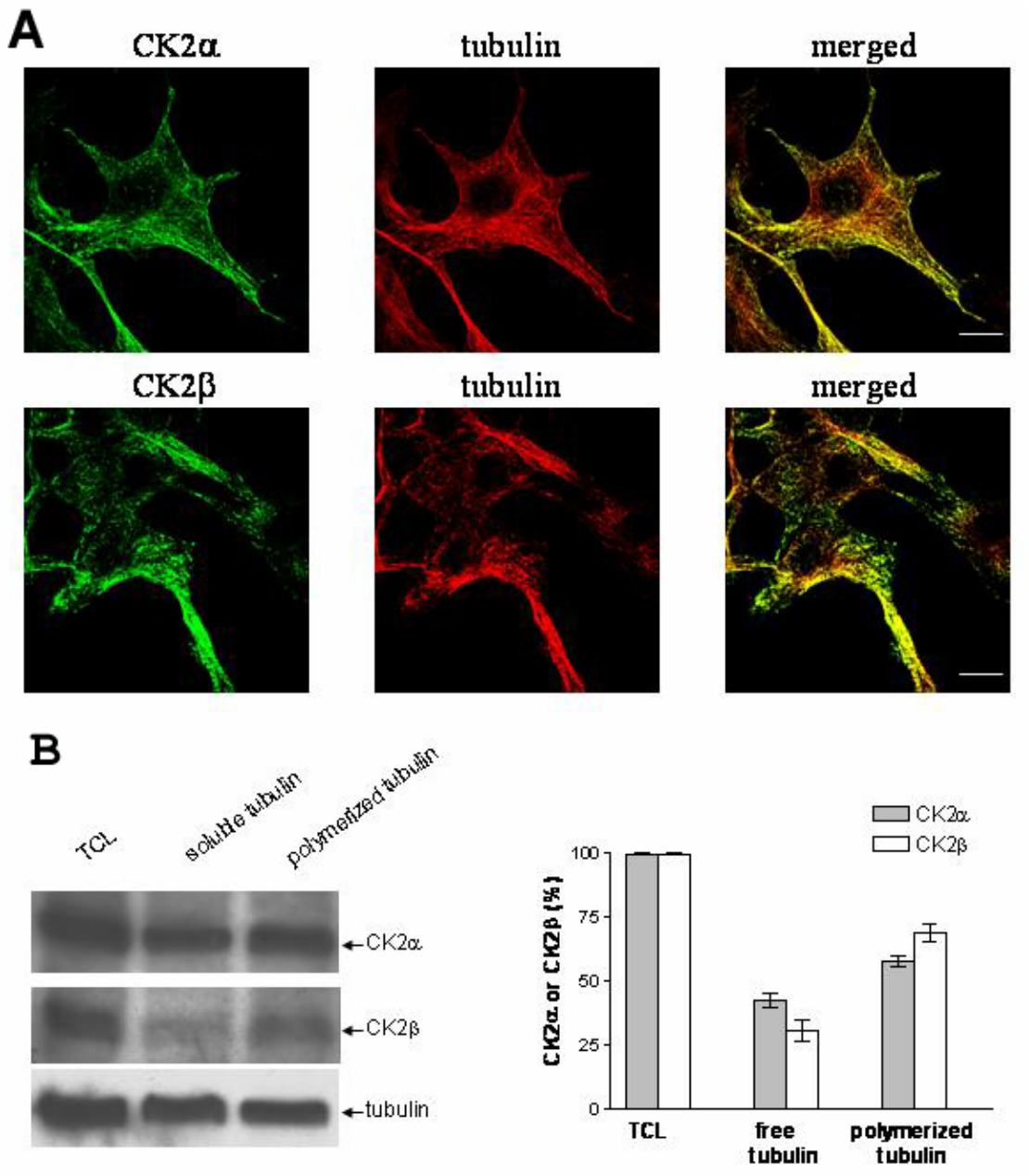

FIG. 2. Cellular localization of CK2α and CK2β to microtubules. (A) COS7 cells were immunostained for confocal microscopic analysis. *Top row*, double-staining of CK2α and β-tubulin; *bottom row*, CK2β and β-tubulin. The secondary antibody used for CK2α/β was Fluor488-conjugated anti-mouse IgG, while tubulin was stained with Fluor594-conjugated anti-rabbit IgG. Bar = 20 μm **(B)** Soluble tubulin heterodimers (free tubulin) and microtubules (polymerized tubulin) were differentially extracted from HeLa cells. Both fractions as well as the total cell lysate (TCL) were analysed by immunoblotting using antibodies as indicated. The histogram shows the relative amounts of CK2α and CK2β in the free and polymerized tubulin fractions. These data are representatives of three independent experiments.

3.2.2.2 CK2 induces microtubule polymerization

We next probed whether CK2 has any effect on microtubule dynamics by using an *in vitro* turbidometric assay for microtubule assembly from purified MAP-free tubulin (Gaskin, 1982). As tubulin polymerizes or depolymerizes in the assay, the turbidity change of the solution is measured. In the absence of CK2, there is minimal self polymerization of tubulin even after prolonged incubation (Figs. 3A & 3B). Addition of CK2 to tubulin at a ratio of 1:240 resulted in substantial polymerization of tubulin into microtubules (Figs. 3A & 3B). Clearly, both the rate and the extent of the polymerization were dramatically enhanced by CK2. When the amount of CK2 was increased, the rate of tubulin polymerization increased in a dose-dependent manner (Figs. 3A & 3B). To verify microtubule formation, rhodamine-labelled tubulin was added into the polymerization mixtures for direct visualization of the assembled microtubules by fluorescence microscopy (Belmont *et al*., 1990). As shown in Fig. 3C, microtubule filaments and bundles were readily observed with the CK2-incubated tubulin, whereas the incubation of tubulin without CK2 showed no obvious microtubule formation. Therefore, in addition to its high affinity for tubulin and microtubules, CK2 also induces the assembly of tubulin into microtubules. Moreover, CK2 appears to cause microtubule bundling, suggesting a strong stabilizing effect on the microtubules.

The holoenzyme of CK2 is a tetrameric complex of two α or α' subunits and two β subunits (Niefind *et al*., 2001). Given the observation that CK2α of the holoenzyme interacts with microtubules, we explored if the microtubule-assembling activity of CK2 is restricted to the holoenzyme by examining the α and β subunits of CK2 individually in the microtubule assembly assay. In contrast to the holoenzyme,

both the α or β subunit induced only very minimal polymerization of tubulin even after prolonged incubation (Fig. 4). Both the CK2α and CK2β-polymerized samples were not markedly different from background tubulin polymerization in the GST-incubated sample. Thus, only the CK2 holoenzyme, but not any of the individual subunits, has the ability to induce microtubule assembly, even though CK2α on its own has microtubule-binding activity.

CK2 has been known to catalyze the phosphorylation of a neural isoform of β-tubulin and some of the MAPs, raising the possibility that it may affect microtubule dynamics through its kinase activity (Diaz-Nido *et al*., 1988; Diaz-Nido *et al*., 1990). Although ATP was not present in the *in vitro* microtubule assembly assay, CK2 is capable of utilizing either ATP or GTP as a phosphate donor in its phosphorylation reactions (Niefind *et al*., 1999). We therefore sought to assess the role of the CK2 kinase activity in inducing microtubule assembly. A kinase-inactive holoenzyme of CK2, in which CK2α was replaced with the kinase-inactive mutant CK2αK68A, was tested in the microtubule assembly assay. Fig. 5 shows that the kinase-inactive CK2 conferred similar microtubule-polymerizing activity as the wild-type enzyme, indicating that the microtubule assembly activity of CK2 is independent of its kinase activity and did not result from the phosphorylation of any microtubule-associated proteins.

Microtubules from brains can be separated into two biochemically distinct pools, namely the “cold labile” and the “cold stable” pools, according to whether they are resistant to cold treatment, which disassembles microtubules (Webb and Wilson, 1980). It was found that CK2 was enriched in the cold stable fraction of the

microtubule preparation from rat brain (Serrano *et al*., 1989). This observation, together with our findings that CK2 associates with microtubules to promote microtubule assembly, prompted us to explore the possibility that CK2 may contribute to the cold stability of microtubules. To test this likelihood, CK2-polymerized microtubules were incubated on ice and the turbidity change was monitored. As a comparison, tau-polymerized microtubules were treated under the same condition (given the fact that tau does not confer the cold stability to microtubules (Baas *et al*., 1994)). As expected, the tau-polymerized sample was depolymerized almost completely within a few minutes (Fig. 6). However, the turbidity of the CK2-polymerized sample was only marginally reduced, even after a prolonged incubation on ice (Fig. 6). This result indicates that CK2 functions to stabilize microtubules against cold-induced disassembly.

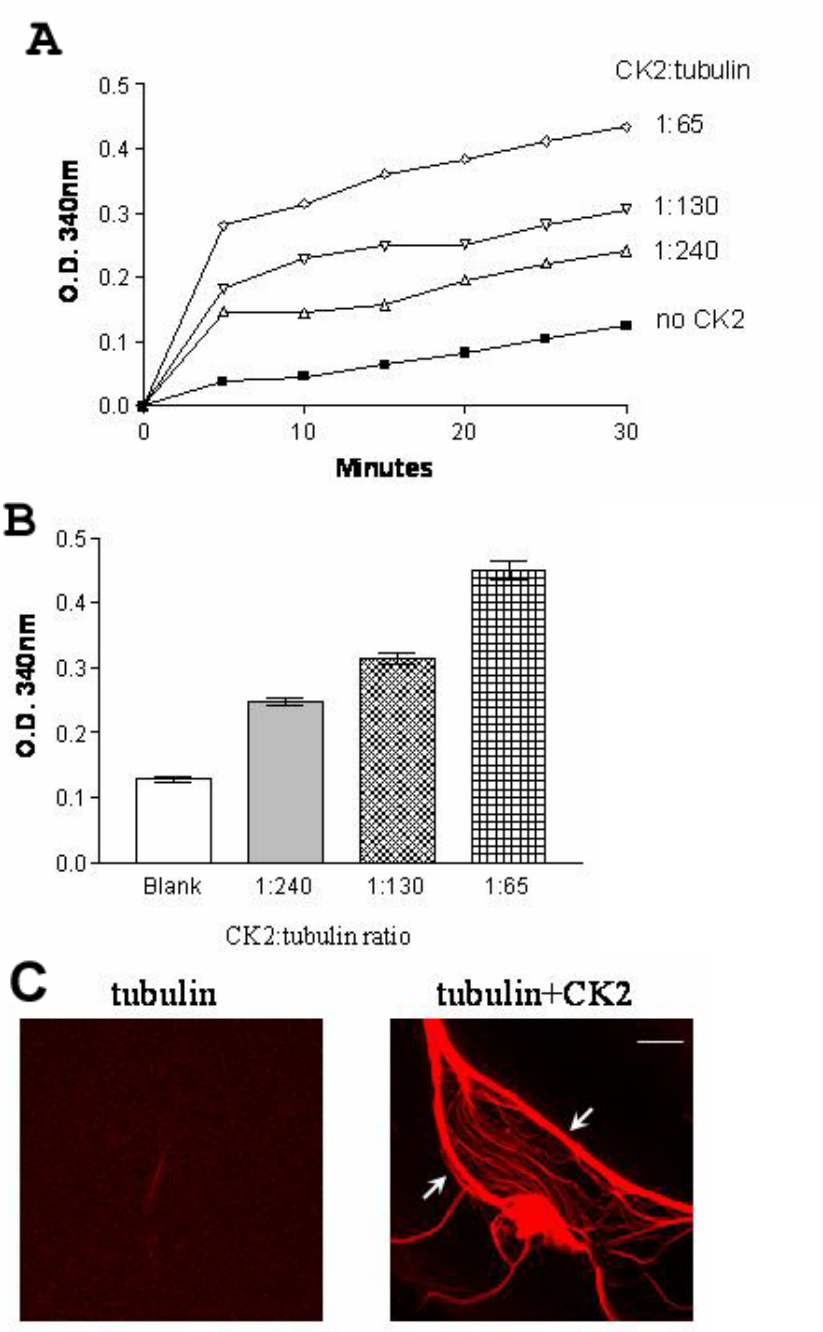

FIG. 3. Effect of CK2 on microtubule assembly. (A) The turbidmetric assay of tubulin polymerization. Microtubule assembly from purified MAP-free tubulin was carried out in the presence of the CK2 holoenzyme at various concentrations (in terms of molar ratios to tubulin). The concentration of tubulin was kept constant in each assay at 2 mg/ml. **(B)** A histogram of the microtubule assembly at various amounts of CK2. The assembly assay was performed as described in (A) for 30 min. The data shown are averages of three separate experiments. **(C)** Fluorescent imaging of microtubules polymerized from a mixture of rhodamine-labelled and unlabelled tubulin (7:1). The tubulin concentration is 2 mg/ml and the CK2 concentration is 62 μg/ml. The *arrows* point to microtubule bundles in the CK2-polymerized sample. Bar = 20 μm

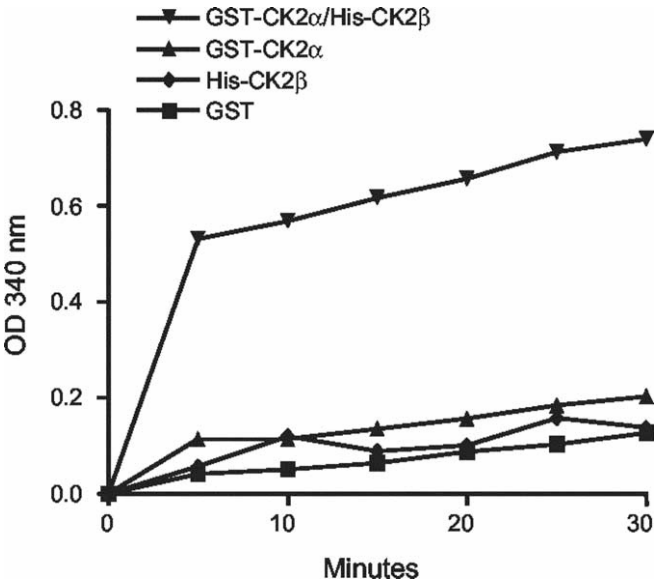

FIG. 4. Microtubule assembly can be induced by the CK2 holoenzyme but not its individual subunits. GST, GST-CK2α and His-CK2β were applied as indicated at 0.1 mg/ml in the microtubule-assembly assay. As a control, the CK2 holoenzyme reconstituted from the same amount of GST-CK2α and His-CK2β as described above was applied. Microtubule assembly was performed at 2 mg/ml of tubulin as described in the *Experimental Procedures*.

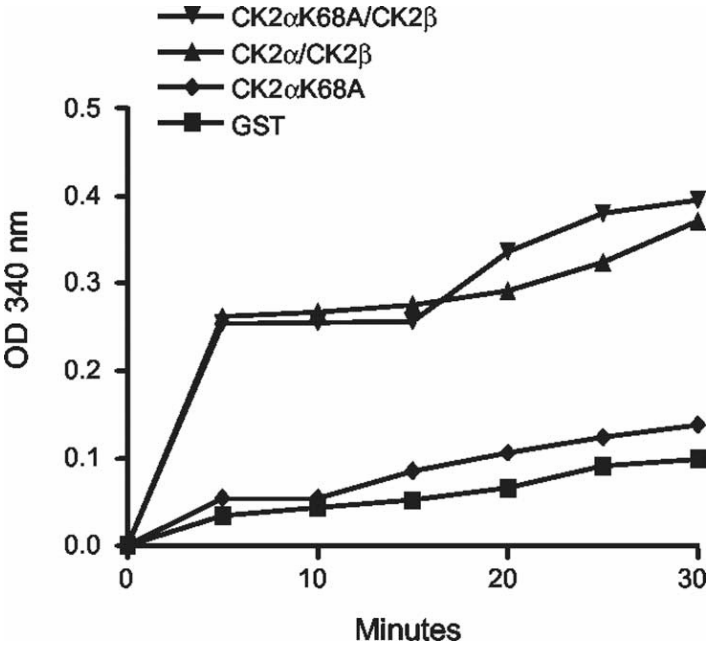

FIG. 5. The kinase activity of CK2 is not required for its function to induce microtubule assembly. The wild-type CK2 enzyme (GST-CK2α/His-CK2β) and the kinase-inactive enzyme (GST-CK2αK68A/His-CK2β) were applied as indicated at 0.1 mg/ml in the microtubule assembly assay. GST-CK2αK68A and GST were also tested at the same amount. Microtubule assembly was performed with 2 mg/ml of tubulin as described in the *Experimental Procedures*.

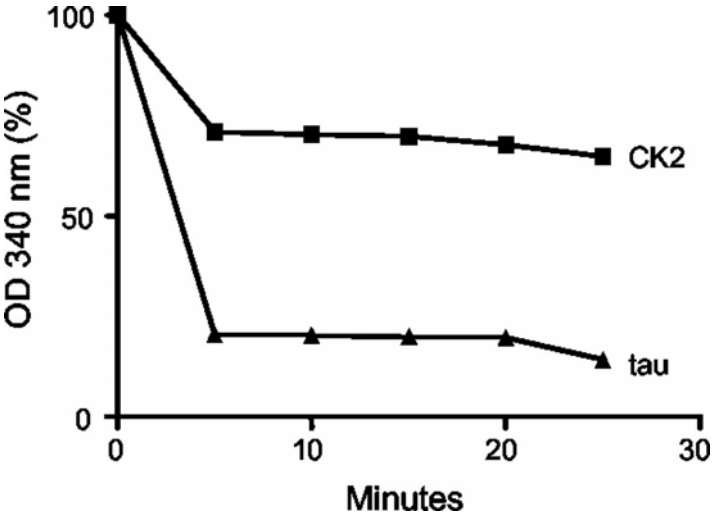

FIG. 6. CK2 confers cold stability to microtubules. Microtubules were polymerized with 0.05 mg/ml of CK2 or 0.16 mg/ml of tau protein for 30 min at 35˚C, where they had attained similar turbidity measurement. The microtubule samples were then incubated on ice and the turbidity measurement was started. The absorbance was expressed as percentages of the measurement when the ice incubation was started.

3.2.2.3 CK2 stabilizes microtubules in vivo

To evaluate the role of CK2 in microtubule dynamics *in vivo*, we knocked down the expression of CK2α/α' in HeLa cells by a gene silencing approach using a siRNA duplex designed based on the human cDNA sequence of CK2α/α' (Sayed *et al*., 2001; Ulloa *et al*., 1993). As shown by the CK2α immunoblot, the introduction of CK2α/α' siRNA into the cells led to a dramatic decrease of the CK2α/α' proteins to a minimal level (approximately 80% decreased) (Fig. 7A). To assess the effect of CK2α/α' knockdown on microtubules, the amounts of cellular microtubules (assembled insoluble tubulin polymers) were determined using the differential extraction method followed by immunoblotting (Lieuvin *et al*., 1994). In addition, the integrity of the cellular microtubule network was examined by immunofluorescent staining and confocal microscopy. The knockdown of CK2α/α' significantly reduced the cellular content of microtubules (Figs. 7A & 7B), suggesting CK2 as one of the factors stabilizing microtubules *in vivo*. We further assessed the microtubule stability using colchicine, a microtubule-disrupting agent. When colchicine was applied at a low concentration (0.2 μM) onto the cells which were transfected with a scrambled siRNA sequence, most of the microtubule structure remained intact (Figs. 7A & 7B). However, such a low dose of colchicine caused severe disruption of the microtubule structure in the CK2α/α'-depleted cells with the microtubule networks collapsed towards the perinuclear region (Fig. 7B). Only negligible amount of microtubules could be extracted from these cells (Fig. 7A). Apparently, the removal of CK2α reduced the stability of cellular microtubules. As a result, it was readily disrupted by colchicine at a very low concentration.

To further substantiate the microtubule-stabilizing function of CK2, we tested whether the microtubule stability could be restored by expression of chicken CK2α in cells where endogenous CK2α/α' had been depleted. As observed with the HeLa cells, knockdown of CK2α/α' in cultured human 293T fibroblasts using the siRNA strongly destabilized the microtubule network, resulting in almost complete disruption of the microtubules by colchicine at 0.2 μM (Fig. 7C). When chicken CK2α was expressed in the 293T cells in which endogenous CK2α/α' was knocked down, the cellular microtubules completely retained their integrity against 0.2 μM colchicine-induced disruption (Fig. 7C). More interestingly, when the kinase-inactive mutant CK2αK68A was expressed, it exhibited the same effect as the wild-type CK2α in rescuing the microtubules from being disrupted by the colchicine treatment (Fig. 7C). These data demonstrate that CK2 is an important mediator of cellular microtubule stability and exerts its effect in a phosphorylation-independent manner.

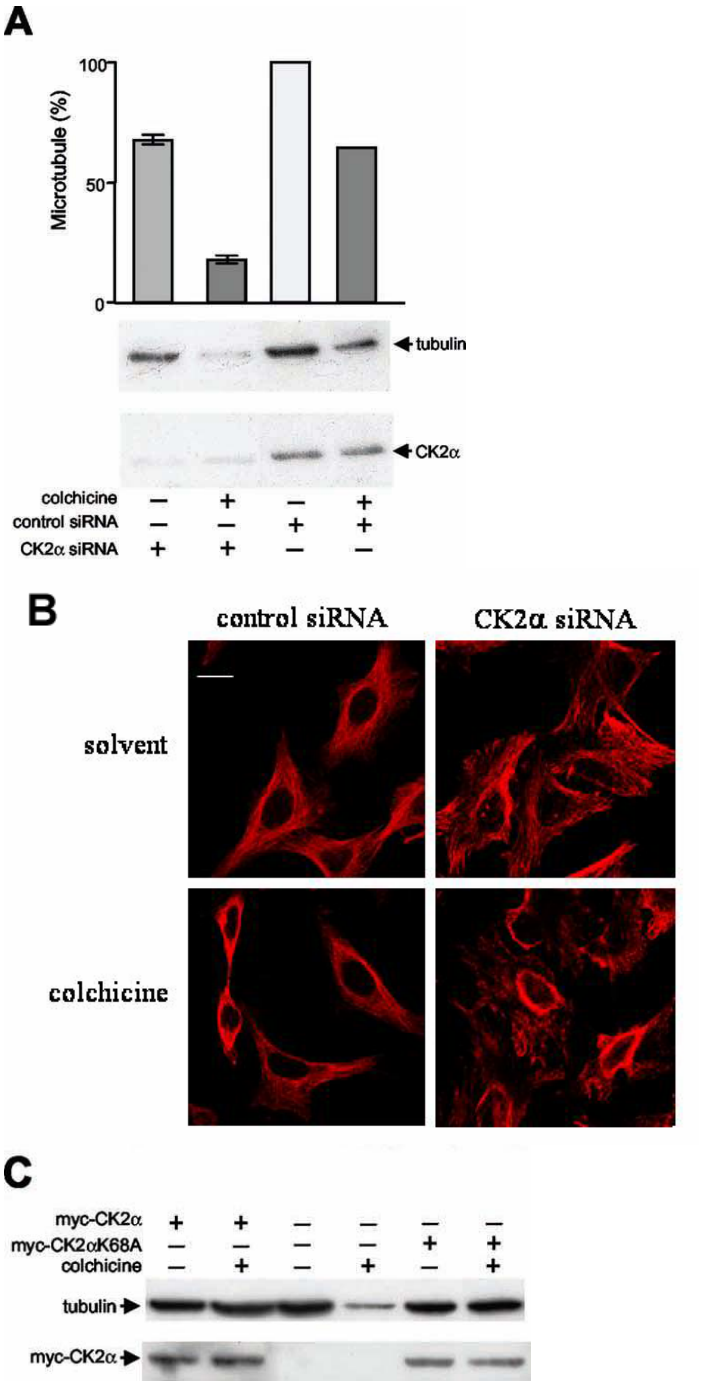

FIG. 7. CK2 stabilizes microtubules *in vivo*. (A) HeLa cells were introduced with siRNA of human CK2α/α' or a scrambled sequence. Knockdown of CK2α was monitored by anti-CK2α immunoblotting. The cells were subsequently treated with 0.2 μM of colchicine or its solvent. Tubulin in the form of polymers (microtubules) was extracted from the cells for β-tubulin immunoblotting. The histogram reflects relative amounts of the microtubules extracted from the cells as indicated to that of the control, which is the sample transfected with the scrambled siRNA sequence and treated without colchicine. The data are representatives of three separate experiments. **(B)** Cells in the experiments described in (**A**) were fixed and stained with the β-tubulin antibody for confocal microscopic imaging. Bar = 20 μm **(C)** Expression of the wild-type or the kinase-inactive mutant of chicken CK2α restored the microtubule stability against the colchicine treatment in CK2α/α'-depleted cells. Prior to treatment of colchicine (0.2 μM), 293T cells were double transfected with siRNA of human CK2α/α' and one of following expression constructs: chicken CK2α, the kinase-inactive mutant of chicken CK2α (CK2αK68A) and the empty vector. Expression of Myc-tagged chicken CK2α and CK2αK68A

was detected by anti-Myc immunoblotting of the cell lysates. Microtubules were extracted using the differential extraction method (see *Experimental Procedures*) for anti-β-tubulin immunoblotting. Representative results of three separate experiments are shown here.

3.2.3 DISCUSSION

In living cells, the microtubule architecture is stabilized by structural MAPs, which associate with microtubules and promote microtubule assembly *in vitro* (Desai and Mitchison, 1997; Mandelkow and Mandelkow, 1995). The evidence presented here identifies CK2 as a structural MAP that mediates microtubule dynamics. We have conducted experiments showing that CK2 is localized to and co-extracted with microtubules. The *in vitro* binding assays demonstrate a direct interaction of CK2 with microtubules as well as tubulin heterodimers. Microtubule-binding sequences are often found in MAPs as repeated sequence stretches enriched in basic amino acids. Although the sequence of CK2α contains some basic regions, there is no typical microtubule-binding motif. Thus, the microtubule association of CK2α suggest the existence of novel microtubule-binding domains within the molecule. In addition, although CK2α also binds p35, both p35 and microtubule are likely to bind CK2α mutually since a p35-CK2 complex interacts physically with tubulin and induces microtubule assembly synergistically (Lim, AC and Qi, RZ, unpublished data).

Structural MAPs such as MAP2 and tau are known to stimulate microtubule assembly from tubulin heterodimers. In our microtubule assembly assays, CK2 exhibited a potent activity in inducing microtubule assembly and bundling from purified tubulin. The physical association of CK2 to microtubules and tubulin heterodimers stimulates both the rate and the extent of microtubule growth. Although CK2α can bind microtubules, the microtubule-assembling and stabilizing function is solely a property of the holoenzyme and neither of the subunits alone can induce polymerization. In addition, CK2-polymerized microtubules display greatly enhanced

stability against the cold treatment, suggesting that CK2 is a strong stabilizer of microtubules. Taken together with the observation that a substantial amount of CK2 exists in the cold-stable microtubules of rat brain (Serrano *et al*., 1989), our findings suggest that CK2 is a new factor endowing cold stability to microtubules. To date, the STOP proteins, doublecortin and BPAG1n3 are the only known MAPs conferring cold stability to microtubules (Denarier *et al*., 1998; Gleeson *et al*., 1999; Guillaud *et al*., 1998; Horesh *et al*., 1999; Yang *et al*., 1999).

Structural MAPs are known to contribute to microtubule stability and distribution within cells (Feng and Walsh, 2001). The finding of CK2 as a structural MAP prompted us to evaluate the regulatory role of CK2 *in vivo* in the microtubule cytoskeleton. The knockdown of CK2α/α' from cells has a strong destabilizing effect on the microtubule architecture. As a result, the microtubule network is very vulnerable and can be readily destroyed by a colchicine insult at 0.2 μM, while such a low concentration of colchicine had no significant effect on microtubules of the cells with intact CK2. Thus, CK2 appears to have an indispensable role in stabilizing cellular microtubules. This is substantiated by the experiment in which chicken CK2α was introduced into the cells to compensate for the loss of the endogenous CK2α/α'. The microtubule instability caused by the deficit of CK2α/α' can be rectified completely by the expression of chicken CK2α, reassuring that CK2 is a vital structural MAP conferring the microtubule stability *in vivo*. It is noteworthy that the removal of CK2α/α' did not by itself cause severe disruption of the microtubules, possibly due to the existence of multiple MAPs other than CK2 in the cells to support the microtubule network.

CK2 is a Ser/Thr protein kinase with a broad substrate spectrum that includes MAP1B and a neural-specific isoform of β-tubulin. We have examined if the kinase activity of CK2 is involved in the microtubule assembly stimulated by CK2. Our *in vitro* assays of microtubule assembly using the kinase-inactive mutant of CK2 indicate that the microtubule-assembling activity of CK2 is independent of its kinase activity. This was corroborated using the CK2α/α'-knockdown cells, in which expression of the kinase-inactive mutant of chicken CK2α completely compensated for the loss of the endogenous CK2α/α', rendering the microtubules resistant to colchicine. With these results, it becomes clear that CK2 imparts a direct regulation of microtubule organization through its physical association with microtubules but not through any enzymatic action. As a multifunctional enzyme, CK2 has been thought to execute its functions through its phosphorylation of a wide range of substrates. The results presented here have revealed a novel CK2 function that is not associated from its intrinsic kinase activity.

It has been proposed that CK2 plays an important role in the maintenance of cell morphology and polarity. Depletion of the catalytic subunits of CK2 in neuroblastoma cells using an antisense approach blocks neuritogenesis (Ulloa *et al*., 1993; Ulloa *et al*., 1994). Pertinent observations also came from yeasts, where the use of temperature-sensitive mutants of CK2α and CK2β demonstrated the importance of these proteins in cell morphogenesis (Rethinaswamy *et al*., 1998; Roussou and Draetta, 1994; Snell and Nurse, 1994). The function described here for CK2 in microtubule dynamics may provide a mechanistic explanation for its role in cell shape control.

Section 4

Summary and Perspectives

4. Summary and Perspectives

Cdk5 is a unique member of the Cdk family of small protein kinases. Despite having 60% sequence identity with Cdk1 and Cdk2, a role for Cdk5 in cell cycle regulation has yet to be identified. Cdk5 is dependent on the association with its protein activator (p35 or p39) for its kinase activity. Although Cdk5 is ubiquitously expressed, its kinase activity is restricted almost exclusively to the nervous system by the neural-specific expression of its regulators p35 and p39 (Ko *et al*., 2001). Though Cdk5 has been shown to play pivotal roles in a variety of cellular processes occurring in the CNS, little is known about the regulation of this kinase. To investigate the regulation and functional properties of Cdk5, we employed the immunoprecipitation methodology, as well as an affinity purification approach to isolate novel p35-binding proteins from rat brain extracts. With the latter approach, the catalytic α subunit of protein kinase CK2 was identified as a novel binding protein of p35 (section 3.1). The evidence presented here suggests that CK2 is a negative regulator of Cdk5-p35 kinase. Based on the results from the protein size exclusion chromatography as well as co-immunoprecipitation assays, CK2α but not the CK2 holoenzyme co-exists with p35 and Cdk5 *in vivo*, though the holoenzyme exhibits a p35-binding capability *in vitro*. CK2 exhibited a strong inhibition on the Cdk5 activation by p35 *in vitro* and *in vivo*. The Cdk5 inhibition is however not associated with CK2 kinase activity since the kinase-dead CK2 mutant displayed an analogous effect as the wild-type protein. Surprisingly, the binding of CK2 to p35 is sufficient to block complex formation between p35 with Cdk5, thereby inhibiting the p35-associated Cdk5 activation. Hence, CK2 and Cdk5 interact with p35 in a mutually exclusive manner and this inhibition effect is reminiscent of the Cdk4/6 inhibition by the INK4 family members (Russo *et al*., 1998; Guan *et al*., 1996; Hirai *et al*., 1995). In addition, it is noteworthy

that cells with higher proliferation rates generally exhibit higher levels of CK2 (Munstermann *et al*., 1990), whereas no Cdk5 activity was found in proliferating neuronal precursors (Tsai *et al*., 1993). Possibly, the presence of CK2 in the proliferating cells helps to maintain Cdk5 activity to a minimal level.

Cdk5 is currently the only functional Cdk found in healthy mature neurons, where it has been implicated in various important cellular activities. There is growing evidence that Cdk5 kinase activity might be elevated during neurodegeneration and deregulation of Cdk5 activity is detrimental to cell survival (Nguyen *et al*., 2001; Patrick *et al*., 1999). The production of p25 (p29 in p39), a truncated form of p35, in neurons correlates with their susceptibility to agents that disrupt calcium homeostasis (Lee *et al*., 2000; Patzke and Tsai, 2002). Calpain, a calcium-dependent protease, induces the conversion of p35 to p25 (Lee *et al*., 2000; Kusakawa *et al*., 2000). Though p25 contains all the elements necessary for Cdk5 binding and activation (Qi *et al*., 1995; Poon *et al*., 1997), its *in vivo* properties are distinct from those of p35 (Patrick *et al*., 1999). Firstly, p25 has a substantially longer half-life than p35 and is able to associate with Cdk5 leading to a sustained activation of the kinase (Patrick *et al*., 1999; Patzke and Tsai, 2002). Secondly, in brain extracts Cdk5-p35 exists in macromolecular complexes displaying little kinase activity while Cdk5-p25 exists as a highly active heterodimer. Thirdly, in contrast to p35, p25 lacks the myristoylation sequence for membrane targeting and therefore is not associated with the plasma membrane (Patrick *et al*., 1999; Patzke and Tsai, 2002). Presumably, the conversion of p35 to p25 relocates Cdk5 from its normal compartments since p25 is concentrated in the cell body and nucleus (Patrick *et al*., 1999). One of the major pathological targets of the Cdk5/p25 complex is the microtubule-associated protein tau. Abnormal

phosphorylation of tau has been implicated in the pathology of several neurodegenerative diseases, such as AD and Parkinson's disease. The expression of p25/Cdk5 in cultured cortical neurons induced hyperphosphorylation of tau, neurite retraction, cytoskeletal abnormalities and apoptosis (Patrick *et al*., 1999). This is further supported by recent studies showing that mice overexpressing p25 caused hyperphosphorylation of tau and neurofilaments, cytoskeletal disruption and behavioral deficits reminiscent of AD (Ahlijanian *et al*., 2000; Noble *et al*., 2003; Cruz *et al*., 2003). Hence, dysregulation of Cdk5 activity may lead to neuronal degeneration.

Neuronal Cdk5 kinase has been implicated in the pathological degeneration of neurons and hence modulating its kinase activity is important. Here, we have identified CK2 as a novel interacting protein, as well as a negative regulator of Cdk5. We have also presented a novel function of CK2 since its inhibitory activity on Cdk5 is not associated with its intrinsic kinase activity. What remains to be determined is the understanding of when and where in the cells CK2 acts to regulate Cdk5 activation. Also the mechanism of how p35 is released from its association with CK2 needs to be established. The binary complexes of p35-CK2 or p35-Cdk5 are stable and cannot be dissociated by introducing Cdk5 or CK2, respectively. CK2 may act as a sentinel molecule in cells to control the Cdk5 activation. Upon dissociation of CK2 from p35, the latter will bind to and activate Cdk5, which in turn catalyses the phosphorylation of p35, promoting the p35 degradation via the proteasome-dependent pathway (Patrick *et al*., 1998). Conceivably, the CK2 association prevents unwanted Cdk5 activation and p35 turnover, thereby preserving the supply of p35. Previously, biochemical separation of brain extract hinted the presence of inhibitory factor(s) in

Cdk5-p35 macromolecular complexes and this inhibitory effect can be liberated when fractionation was performed in the presence of 10% ethylene glycol (Lee *et al*., 1996a). Preliminary investigation showed that the elution profiles of Cdk5, p35 and CK2α were unaffected by the presence of ethylene glycol, but surprisingly, CK2β co-eluted together with Cdk5, p35 and CK2α at the void volume of the column under this new condition (Lim AC and Qi RZ, unpublished result). We showed that Cdk5 binds to the catalytic subunit but not the holoenzyme of CK2 *in vitro*. Possibly under normal conditions, CK2α that is devoid of its regulatory β-subunit interacts physically with Cdk5, thereby inhibiting the formation of Cdk5-p35 kinase. The presence of ethylene glycol may have caused a distinctive change in the elution profile of CK2β, and in so doing liberated CK2α from some of its binding proteins including Cdk5 and p35 to form the holoenzyme with CK2β. Hence, it is possible that endogenous Cdk5 monomer may then be activated by adding its exogenous activator.

In addition to CK2α, a list of other Cdk5/p35-interacting proteins was also identified and these proteins can be mainly classified into two different categories. The former includes cytoskeletal and cytoskeleton-associated proteins. Cdk5 is the only kinase that affects the electrophoretic mobility of human NF-H and is thought to be the major neurofilament kinase (Kesavapany *et al*., 2003b). Cdk5 is involved in crosstalk with other signal transduction pathways such as the MAPK and the myelin-associated glycoprotein pathways to influence the phosphorylation of neurofilaments and other cytoskeletal proteins. Both the hyperactivation of Cdk5 activity and the subsequent hyperphosphorylation of neurofilaments and tau protein have been implicated in the pathogenesis of neurodegenerative disorders such as AD and amyotrophic lateral sclerosis. Drebrin is an actin-associated protein where it co-

localizes with the actin filaments and has been suggested to regulate the assembly and disassembly of actin filaments (Shirao *et al*., 1994; Asada *et al*., 1994). Previously, p39 has been reported to bind actin and hence, Cdk5 may play a role in the modulation of the cellular actin dynamics. The interaction between p35 with α- and β-tubulin may have a role in the regulation of cellular microtubule dynamics. The latter group of p35-binding proteins isolated includes proteins that are involved in signal transduction and other neuronal processes. Coincidentally, some of these proteins have been reported previously to be Cdk5/p35-interacting proteins (Table A & B).

Microtubule dynamics is essential for many vital cellular processes such as morphogenesis and motility. Previous reports have established the co-existence of Cdk5-p35 kinase with microtubule in brain extract (Sobue *et al*., 2000; Paudel *et al*., 1993). Furthermore, we have isolated CK2 as a p35-binding protein and previous studies have also implicated CK2 in regulating of microtubule cytoskeleton reorganization (Serrano *et al*., 1987; Diaz-Nido *et al*., 1988; Serrano *et al*., 1989). These few lines of evidence prompted us to explore the involvement of CK2 complex in mediating microtubule dynamics. We showed that the CK2 holoenzyme interacts directly with both microtubules and tubulin heterodimers through its catalytic subunit (section 3.2). Though CK2α does not have any typical microtubule-binding motif, its sequence contains some basic regions, reminiscent of new microtubule-binding domains. We demonstrated that the CK2 holoenzyme, but neither of its individual subunits, exhibited a potent activity of inducing microtubule assembling and bundling. Moreover, CK2-polymerized microtubules were strongly stabilized against cold-induced depolymerization, suggesting CK2 as a new factor in endowing the cold stability on microtubules. Interestingly, CK2 enzymatic activity is not necessary for

its microtubule assembling and stabilizing function since a kinase-dead mutant displays analogous activity as the wild-type protein. Given the fact that CK2 is a serine/threonine protein kinase with a myriad of substrates (Meggio and Pinna, 2003), we have presented a novel CK2 function that is independent to its intrinsic kinase property. *In vivo*, CK2 has a strong stabilizing effect on the microtubule architecture since knockdown of its catalytic subunit renders the cells very vulnerable to destruction by low concentration of colchicines insults. Microtubule instability can be rectified completely by the expression of chicken CK2α in CK2α-depleted cells, substantiating the notion that CK2 is a vital structural MAP that confers microtubule stability *in vivo*. Several studies have indicated that CK2 is instrumental and necessary in cell survival (Ahmed *et al*., 2002; Pinna, 2002; Litchfield, 2003). In this regard, CK2 has been postulated to play a central role in protecting cells against stress-induced apoptosis. Taken together, our observation that CK2 confers protection against colchicine-induced disruption of microtubule network implies that CK2 may be a cell survival molecule.

It remains to be determined if the effects and mechanism reported here are important in neuronal cells. Observations from our laboratory showed that p35 is also associated and co-localized with the cellular microtubule network. Since both p35 and CK2 have direct effect on microtubule dynamics independently, it would be of further interest to define if a p35-CK2 complex may enhance or have further effect on the cellular microtubule architecture. Our preliminary results show that this protein complex has a major direct effect on enhancing microtubule polymerization synergistically *in vitro* (Lim AC and Qi RZ, unpublished result). Seemingly, the physical association between p35 and CK2 may serve more than one function, though

this is yet to be further explored. Possibly, p35 may play independent roles with CK2 at "the right place and the right time". The sequence of CK2α contains several basic regions, reminiscent of the typical nuclear localization sequence (NLS). Although no nuclear import of CK2α has been reported, CK2α has been observed to be localized to the nucleus (Filhol *et al*., 2003), hinting a possible role for these basic regions in the localization of CK2α. Analogous to Cdk5-p35, CK2 is a protein kinase that brings about phosphate transfer reactions in order to regulate various cellular functions. CK2 binds Cdk5/p35 and down-regulates the activation of Cdk5 by p35. It remains to be determined what complementary effects Cdk5 and/or p35 may have on CK2 in the cells. Since CK2 interacts with tubulin as well as p35, it would also be desirable to fine map the regions on CK2 that interacts with either proteins. More importantly, the significance and detailed molecular mechanisms underlying the interactions between CK2 with p35 and tubulins, respectively, remain to be further elucidated.

Immense progress has been made in identifying the regulatory mechanisms involved in Cdk5 signaling. Recent results have broadened our perspective and include more variables in our perception of the cellular functions of Cdk5. Moreover, there is growing evidence implying that aberrant regulation of Cdk5 leads to neurodegeneration and cell death. Hence, instead of identifying a conclusive regulatory mechanism for this enzyme, its network of signaling mechanisms is much more complex than expected.

Section 5

References

5. References

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Section 6

Appendix

6.1 Tables

6.2 Publications

Section 6 - Part I

6.1 Tables

Table A. Proteins associated with Cdk5 and p35

Associated Protein	Putative function	Reference(s)
Cables	enhances *c*-Abl's association and phosphorylation of Cdk5 at Tyr15	(Zukerberg *et al*., 2000)
Fyn	promotes Cdk5 activity through phosphorylation of Tyr15	(Sasaki *et al*., 2002)
SET	enhances Cdk5-p35 activity	(Qu *et al*., 2002)
C42	inhibits Cdk5 activation by p35	(Ching *et al*., 2002)
Ribosomal protein L34	inhibits Cdk5-p35 activity	(Moorthamer and Chaudhuri, 1999)
DbpA	inhibits Cdk5-p35 activity	(Moorthamer *et al*., 1999)
Ubiquitin	proteasomal degradation of p35	(Patrick *et al*., 1998)
Rac	phosphorylation of PAK1	(Nikolic *et al*., 1998; Rashid *et al*., 2001)
PAK1	phosphorylation of PAK1	(Nikolic *et al*., 1998; Rashid *et al*., 2001)
Actin	potential role in actin dynamics	(Humbert *et al*., 2000a)
α-actinin1	potential role in the localization of Cdk5 to the synaptic cytoskeleton	(Dhavan *et al*., 2002)
NFH/NFM	phosphorylation of NFH/NFM	(Lew *et al*., 1994; Grant *et al*., 2001)
Tau	phosphorylation of tau	(Ishiguro *et al*., 1994; Baumann *et al*., 1993)
Munc 18	phosphorylation of Munc 18	(Fletcher *et al*., 1999; Shuang *et al*., 1998)
Syntaxin 1A	mediates trafficking and secretion	(Shuang *et al*., 1998)
Amphiphysin 1	phosphorylation of amphiphysin 1	(Floyd *et al*., 2001)
CaMKIIα	regulation of synaptic plasticity	(Dhavan *et al*., 2002)
β-Catenin	regulates N-cadherin-mediated cell adhesion and the association of β-catenin with presenilin 1	(Kwon *et al*., 2000;

		Kesavapany *et al*., 2001)
PP1	phosphorylation of PP1.I-2	(Agarwal-Mawal and Paudel, 2001)
pRb	phosphorylation of pRb	(Lee *et al*., 1997)
PCTAIRE1	phosphorylation of PCTAIRE1	(Cheng *et al*., 2002)
Lipofuscin	putative pathogenic process of ALS	(Bajaj *et al*., 1998)
ErbB	mediates neuregulin-induced AChR expression at neuromuscular junction	(Fu *et al*., 2001)
C48	not known	(Ching *et al*., 2000)
C53	not known	(Ching *et al*., 2000)
IC53	human homologue of rat C53 and potential role in cell proliferation	(Chen *et al*., 2002)
IC53-2	human homologue of rat C53 and potential role in cell proliferation	(Xie *et al*., 2003a)
Cyclin D	Cdk5 binding protein and potential regulator of Cdk5	(Xiong *et al*., 1992)
Cyclin E	Cdk5-binding protein and potential regulator of Cdk5	(Miyajima *et al*., 1995)
Ik3-2	homologue of Cables (ik3-1)	(Sato *et al*., 2002)
Puralpha	developmentally timed DNA replication	(Khalili *et al*., 2003)
Cdk5/p35-regulated kinase (CPRK)	phosphorylates and regulates CPRK activity	(Kesavapany *et al*., 2003a)
Nestin	phosphorylates nestin	(Sahlgren *et al*., 2003)
Apoptosis-associated tyrosine kinase (AATK)	phosphorylation of AATK	(Honma *et al*., 2003)

Table B. Cdk5 substrates

Cdk5 Substrate	Putative function of phosphorylation	Reference(s)
p35	suppresses the proteolytic conversion of p35 to p25 by calpain and facilitates the proteasomal degradation of p35	(Patrick *et al*., 1998)
Cables	mediates its interaction with Cdk5	(Zukerberg *et al*., 2000)
PAK1	inhibits PAK1 activity and regulates cytoskeletal dynamics	(Nikolic *et al*., 1998; Rashid *et al*., 2001)
NFH/NFM	regulates axonal transport of neurofilaments	(Grant *et al*., 2001; Ackerley *et al*., 2003)
Tau	regulates microtubule binding and dynamics	(Baumann *et al*., 1993)
MAP1B	regulates microtubule stability	(Pigino *et al*., 1997; Paglini *et al*., 1998)
Nudel	regulates dynein-mediated axonal transport	(Niethammer *et al*., 2000)
Synapsin 1	regulates synaptic transmission	(Matsubara *et al*., 1996)
Munc 18	mediates the interaction between Munc 18 and syntaxin 1A	(Shuang *et al*., 1998)
Amphiphysin 1	regulates synaptic vesicle endocytosis and neurite outgrowth	(Floyd *et al*., 2001; Tomizawa *et al*., 2003)
Dynamin 1	facilitates synaptic vesicle endocytosis	(Tan *et al*., 2003; Tomizawa *et al*., 2003)
NR2A (N-methyl-*D*-aspartate receptor)	regulates synaptic transmission, plasticity and hippocampal CA1 cell death	(Li *et al*., 2001; Wang *et al*., 2003)
Src	regulates functions of Src in neurons	(Kato and Maeda, 1999)
β-APP	mediates APP localization and function	(Iijima *et al*., 2000)
β-catenin	regulates N-cadherin-mediated cell adhesion and the association of β-catenin with presenilin 1	(Kwon *et al*., 2000; Kesavapany *et al*., 2001)
Presenilin 1	regulates PS1 stability and metabolism	(Lau *et al*., 2002)
P/Q type calcium channel	inhibits the neurotransmitter release in synaptic transmission	(Tomizawa *et al*., 2002)
DARPP-32	regulates dopamine signaling and the	(Bibb *et al*.,

	stimulant action of caffeine	1999; Lindskog *et al*., 2002)
MEK1	inhibition of MEK1 activity	(Sharma *et al*., 2002)
JNK3	inhibits JNK3 activity and mediates neuronal apoptosis	(Li *et al*., 2002)
PP1 inhibitors I-1/I-2	activates I-1 and I-2 to mediate PP1 activity	(Agarwal-Mawal and Paudel, 2001; Huang and Paudel, 2000)
pRb	mediates neuronal apoptosis	(Lee *et al*., 1997)
p53	mediates p53 transcriptional activity	(Zhang *et al*., 2002b)
MEF2	inhibits MEF2 transcriptional activity and mediates neuronal apoptosis	(Gong *et al*., 2003)
Dab1	mediates neuronal migration	(Keshvara *et al*., 2002)
PCTAIRE1	promotes the PCTAIRE1 activation	(Cheng *et al*., 2002)
ErbB	mediates neuregulin signaling at the neuromuscular junction	(Fu *et al*., 2001)
ErbB2/ErbB3	regulates neuregulin-induced Akt activity and neuregulin-mediated neuronal survival	(Li *et al*., 2003)
Outer Dense Fibers	Regulation of sperm tail development	(Rosales *et al*., 2003)
FAK	mediates microtubule organization, nuclear movement and neuronal migration	(Xie *et al*., 2003b)
Cdk5/p35-regulated kinase (CPRK)	regulates CPRK activity	(Kesavapany *et al*., 2003a)
Nestin	regulates organization of nestin and its association with p35	(Sahlgren *et al*., 2003)
P_γ (retinal cGMP phosphodiesterase)	mediates PDE activity and phototransduction	(Hayashi *et al*., 2000; Matsuura *et al*., 2000)

Section 6 - Part II

6.2 Publications

6.2 Publications

The marked (*) references are presented in this thesis

1. **Lim, A. C.**, and Qi, R. Z. (2003) Cyclin-dependent kinases in neural development and degeneration. *J Alzheimers Dis.* **5**, 329-335.

2. **Lim, A. C.**, Qu, D., and Qi., R. Z. (2003) Protein-protein interactions in Cdk5 regulation and functions. *NeuroSignals.* **12,** 230-238.

3. ***Lim, A. C.**, Tiu, S. Y., Li, Q., and Qi, R. Z. (2004) Direct regulation of microtubule dynamics by protein kinase CK2. *J Biol Chem.* **279,** 4433-4439.

4. ***Lim, A. C.**, Hou, Z., Goh, C. P., and Qi, R. Z. (2004) Protein kinase CK2 is an inhibitor of the neuronal Cdk5 kinase, *Accepted in the J Biol Chem.*.

Conference Papers

1. **Lim, A. C.**, Goh, C-P., Tiu, S-Y., and Qi, R.Z. (2002). Regulation of Cdk5/p35 kinase by direct interaction with p35BP. *Anti-ageing Research Conference*, Singapore. [Poster Abstract].

2. **Lim, A. C.**, Goh, C-P., Tiu, S-Y., and Qi, R.Z. (2002). Regulation of Cdk5/p35 kinase by direct interaction with p35BP. *42nd Americian Society for Cell Biology Meeting*, San Francisco, California, USA. [Poster Abstract].

3. **Lim, A. C.**, Goh, C-P., Tiu, S-Y., Wong, B-S., and Qi, R.Z. (2003). Identification of a neuronal Cdk5 activator-binding protein as Cdk5 inhibitor. *33rd Soc Neurosci Meeting*. New Orleans, Louisiana, USA. [Poster Abstract].

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Cyclin-dependent kinases in neural development and degeneration

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Abstract. There is increasing evidence suggesting that cyclin-dependent kinases (Cdks) that normally regulate cell cycle progression may also be involved in the pathogenesis of neurodegenerative disorders and in the apoptotic death of neurons subjected to various insults. Deregulation of Cdks has been observed in an increasing number of neurological disorders, including Alzheimer's and Parkinson's diseases as well as amyotrophic lateral sclerosis (ALS). Unchecked expression of these proteins can potently induce apoptotic or necrotic neuronal cell death. Cdks initiate death pathways by derepressing E2F-1/pRb-dependent transcription at neuronal G1/S checkpoint. On the contrary, deregulation of Cdk5, which is not involved in cell cycle control, contributes to neurodegeneration by altering the phosphorylation state of non-membrane-associated proteins. This review describes work indicating Cdks' roles in the nervous system and how they may cogitate in leading neurons to their demise.

Keywords: Cyclin-dependent kinases, cell cycle, neuronal development, degeneration

1. Introduction

The regulation of cell cycle progression is a tightly controlled process. The timing through the various phases of G1/S/G2 and M is mediated through an ordered progression of Cdk activation [1,2]. Cyclin-dependent kinases (Cdks) are proline-directed serine/threonine kinases whose activities are controlled through a complex series of mechanisms, including binding to their appropriate cyclin partners, activating and inactivating phosphorylation modifications, and endogenous inhibitors of the Cdk activity [2,3]. In general, each Cdk periodically interacts with a specific subset of cyclins to regulate the Cdk activity. There are at least nine different Cdks (Cdk1-Cdk9) and many more cyclins (cyclin A through T). Cyclin/Cdk complexes are in turn regulated in defined stoichiometric combination with specific small inhibitory proteins, the Cdk inhibitors (CKIs). There are two families of CKIs: the INK4 (inhibitor of Cdk4) family members, $p16^{ink4a}$, $p15^{ink4b}$, $p18^{ink4c}$ and $p19^{ink4d}$, specifically inhibit cyclin D-associated kinases, and the KIP (kinase inhibitor protein) family members, $p21^{cip1/waf1}$, $p27^{kip1}$ and $p57^{kip2}$, bind and inhibit the activity of cyclin E/Cdk2, cyclin A/Cdk2 and cyclin B/Cdk1 complexes [4,5].

In contrast to cell cycle progression, cell death entails a different setup. Cell death can occur through a spectrum of both necrotic and genetically regulated processes. The former is associated with the release of cellular components and inflammation while the latter (apoptosis) proceeds through highly controlled mechanisms [6]. Apoptosis, or programmed cell death, is a fundamental and essential process in the development and tissue homeostasis of multicellular organisms. It is particularly important for the proper development of highly differentiated organs such as the brain and other parts of the nervous system. It was reported that half of all the neurons produced during neurogenesis die apoptotically before the nervous system matures [7]. In addition, apoptosis is also involved in various neurodegenerative disorders such as Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS).

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Though cell cycle and apoptosis are quite different processes, there is evidence that suggests they do share common connections [8]. During both processes, cells lose attachment and volume, condense their nuclei, disassemble the nuclear lamina, and display membrane blebbing. Furthermore, cell cycle and apoptosis share some common participants including pRb, E2F and p53. A growing body of evidence has indicated that deregulation of the cell cycle can either directly trigger apoptosis or increase sensitivity to apoptotic inducers [8,9]. Although neurons are in a state of terminal differentiation and incapable of undergoing cell division, nonetheless they retain certain elements of the cell cycle machinery and have the capability of reactivating aspects of the replicative mechanism when under stress. Here, we discuss the different aspects and roles of the Cdks and their participation in neural development and degeneration.

2. Cell cycle Cdks in neural development

Cdks are pivotal cell cycle mediators that regulate proliferation, differentiation, senescence and apoptosis [10]. Neural development involves proliferation of the precursor cells, cell fate determination, as well as apoptosis. Cell cycle Cdks are crucial regulators of proliferation in the precursor stage; their activities are being blocked by sophisticated mechanisms including the binding of CKIs once the cells start differentiation; and eventually in the differentiated cells, these Cdks are disappeared. Cdks appear to mediate cell cycle progression by phosphorylating a panel of key substrate targets. An important and well-characterized target of Cdks is the retinoblastoma protein (pRb). In mid-to-late G1, pRb is phosphorylated by cyclin D1/Cdk4/6 and cyclin E/Cdk2 complexes. This phosphorylation inhibits the negative influence of pRb on the E2F transcription factors, which then proceed to induce expression of genes controlling DNA replication and subsequently cell cycle progression [11]. By using dominant negative mutants of Cdks, Ferguson and co-workers showed that both Cdk2 and Cdk4/6 are crucial for cell cycle regulation in neural precursor cells [12]. Moreover, growth arrest induced by the Cdk4/6 mutants is pRb-dependent, highlighting the importance of the pRb regulatory pathway in neuronal development and cell cycle regulation [12].

Neural development requires a strict control of the Cdk activities to mediate the entry into, as well as the withdrawal from the cell cycle. The Wee kinases block the entry into mitosis by phosphorylating and inhibiting the activity of the mitotic kinase, Cdk1. The unique temporal and spatial patterns of the expression of various *Xenopus* Wee kinases were implied to execute roles in the coordination of neural tissue development [13]. *Xenopus* Wee1 is expressed only as a maternal gene product; *Xenopus* Wee2 is predominantly a zygotic gene product; while a third Wee kinase, *Xenopus* Myt1, is both a maternal and a zygotic gene product. In accord with the changing levels of these Cdk inhibitory kinases, the pattern of embryonic cell division becomes asynchronous and spatially restricted in the *Xenopus* embryo. Intriguingly, at the onset of zygotic transcription, Wee2 is expressed in regions of the embryo that are devoid of mitotic cells whereas Myt1 is expressed in regions of the embryo that have high levels of proliferation [13].

Another regulatory mode of Cdk activities is through the CKIs. By *insitu* hybridization of mouse embryos, members of the INK4 family, $p18^{ink4c}$ and $p19^{ink4d}$, but not $p16^{ink4a}$ and $p15^{ink4b}$, were shown to be expressed in the central nervous system (CNS) during mouse brain development [14]. Expression of $p18^{ink4c}$ in developing brain is restricted to the dividing neuroblasts and not the differentiating postmitotic neurons. Likewise, $p18^{ink4c}$ was expressed precisely at those developmental stages when neuroblasts switch from a symmetric to an asymmetric pattern of cell division. In contrast, $p19^{ink4d}$ expression was seen in the dorsal root ganglia, spinal cord, and focally throughout the brain, but primarily in the postmitotic neurons [14]. To further support the notion that there is a region-specific expression of CKIs in the brain, Legrier and co-workers exemplified that a number of cell cycle inhibitors are expressed in the adult brain either in a ubiquitous fashion (as $p19^{ink4d}$) or in specific brain regions ($p15^{ink4b}$ in the forebrain, $p27^{kip1}$ and $p21^{cip1/waf1}$ in the cerebellum) [15,16]. In contrast, $p18^{ink4c}$ expression was detectably only in the highly neurogenic olfactory epithelium [15]. Interestingly, $p19^{ink4d}$ and $p27^{kip1}$ were found in mature neurons of the neocortex and hippocampus, suggesting a dormant cell cycle that potentially can be activated under appropriate circumstances [16]. Indeed, the expression of CKIs in neurons correlates with their withdrawal from the cell cycle [17, 18].

3. Cell cycle Cdks and neurodegeneration

There is increasing evidence suggesting that Cdks may have functions beyond the cell cycle regulation.

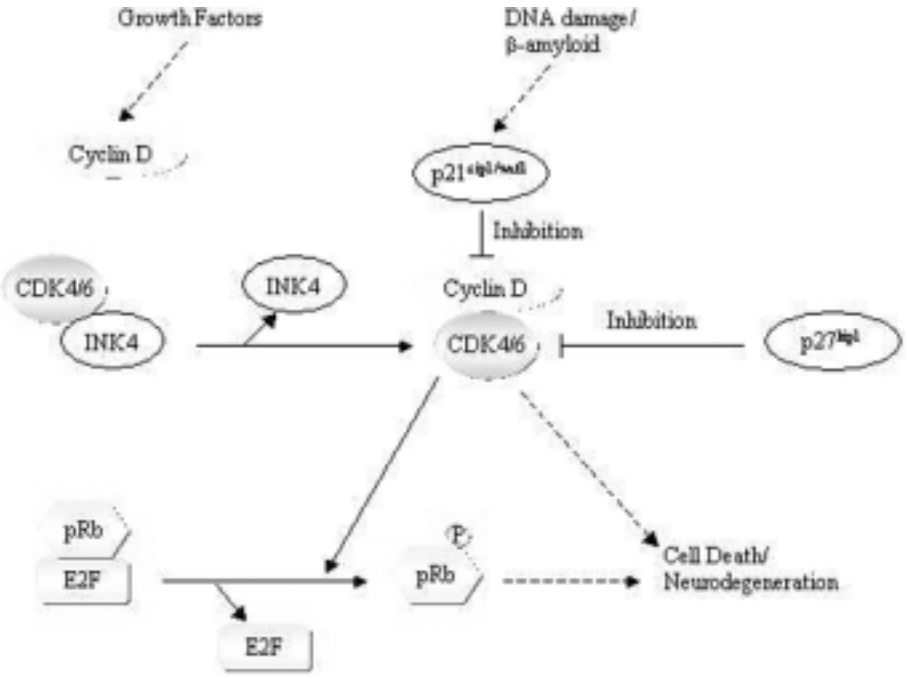

Fig. 1. A schematic overview of the involvement of cell cycle Cdks in cell death and neurodegeneration.

A number of reports have indicated the requirement of Cdk signals for the death of cultured postmitotic neurons that are exposed to certain death insults. Inappropriate cyclin B and cyclin D1 transcripts have been observed in neuronal PC12 cells and sympathetic neurons deprived of trophic support [19,20]. In addition, the cyclin D1-associated kinase activity, as well as the phosphorylation of its substrate pRb, increases during the death of cultured embryonic neurons that have been exposed to DNA damage [21] or amyloid-β [22], the latter being a major toxic component in AD (Fig. 1). In addition, using transgenic mice expressing a mutant superoxide dismutase (SOD1G37R) associated with ALS, Nguyen and co-workers showed an upregulation and mislocalization of Cdk4 in the motor neurons of these mice. The elevated Cdk4 activity is associated with increased expression of nuclear Cdk4 and cyclin D1 as well as the abnormal phosphorylation of pRb protein at the Cdk phosphorylation sites [23].

Previously, apoptotic death was thought to be exclusively related to the process of neuronal selection during the CNS development. It is certainly unexpected that a repertoire of events typical of proliferating cells might be activated in degenerating neurons. The induction of Cdks in neurons is associated with their dedifferentiation and triggered neuronal death, which can be rescued by the use of Cdk inhibitors or the expression of dominant-negative forms of the kinases [24,25]. Taken together, inappropriate activation of cell cycle signal, in particular Cdk4/6-pRb pathway, in the environment of differentiated postmitotic neuron may lead to death.

4. Cdk5: a Cdk that isn't cyclin-dependent

Cdks are a family of small protein kinases that share greater than 40% identity and the activation of Cdks requires the binding of cyclin [2]. Cdk5 is a unique member of this family. It was originally discovered by virtue of its sequence homology to the cell cycle Cdks [26]. Despite having 60% sequence identity with Cdk1 and Cdk2, a role for Cdk5 in cell cycle regulation has yet to be identified. Cdk5 is dependent on the association with two protein activators for its kinase activity. The first activator of Cdk5 was isolated from mammalian brains and subsequently cloned as a 35-kDa protein, now known as p35 [27,28]. Another regulator of Cdk5 (p39) was identified by its sequence homology to p35, with which it shares 57% amino acid identity [29]. The Cdk5 kinase activity is restricted virtually exclusively to the nervous system by the neural-specific expression of p35 and p39 [27,29], though recent observations showed it can also be located in other cell types [30–32]. Despite their distinctiveness, struc-

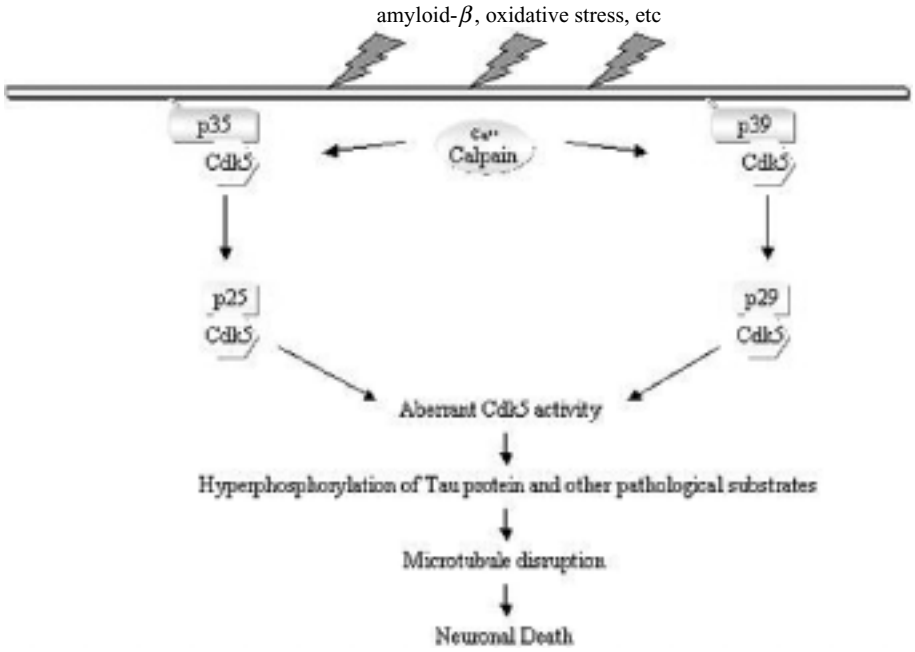

Fig. 2. Cleavage of p35 and p39 is neurotoxic. p35/p39 targets Cdk5 to the membrane, where it phosphorylates several substrates. Calpain, a calcium-dependent protease, can be activated by oxidative stress and the β-amyloid peptide and it converts p35 and p39 to p25 and p29, respectively. p25/p29 lacks the myristoylation signal and this leads to aberrant Cdk5 activity, which may results in neuronal death.

tural studies suggest that p35 takes up a cyclin-like fold that interacts with Cdk5 [33].

The regulation of kinase activity by phosphorylation is also different for Cdk5. Though the activation of the authentic Cdks, Cdk1 and Cdk2 requires phosphorylation of Thr161 and Thr160 respectively in the T-loop, this step is dispensable for the Cdk5 activation [33,34]. Furthermore, phosphorylation of Tyr15 by Wee1/Myt1 dual-specific kinases inhibits most Cdk family members, but not Cdk5 [35]. Interestingly, its activity increases upon phosphorylation at Tyr15 by the enzymes Abl and Fyn [36,37]. In addition, Cdk5 activity is not affected by any members of the CKIs [38], which further substantiates Cdk5 as a unique member of the Cdk family.

5. Cdk5 in CNS development

Cdk5 is currently the only functional Cdk found in healthy mature neurons of the CNS. During neural differentiation, the expression of cell cycle Cdks is diminished while Cdk5 (and its activators) becomes the most abundant Cdk [39]. Gene targeting experiments showed a fundamental role of Cdk5 in CNS development. Mice that are deficient of *Cdk5* die just before or after birth and exhibit widespread disruptions in neuronal layering of many brain structures, possibly due to impairment in neuronal migration [40]. Expression of the Cdk5 transgene under the p35 promoter in *Cdk5*-deficient mice rescues the defects in the nervous system of the *Cdk5*-null phenotype, and further demonstrates that Cdk5 activity is necessary for normal development of the nervous system [41].

Mice that lack p35 show an inverted layering of cortical neurons, a mild similarity to that seen in $Cdk5^{-/-}$ mice. In contrast to the *Cdk5*-null mutants, these mice are viable and fertile, although they are particularly susceptible to seizures and early lethality [42]. Disruption of the p35 gene is not lethal due to compensation of the other activator, p39 [43]. While the *p39*-deficient mice do not show any noticeable defects, the phenotype of the *p35/p39* double-mutant mice is indistinguishable from that of the *Cdk5*-null mice [43]. Thus, p35 and p39 are probably the only essential activators of Cdk5 in the brain, and p35 and p39 have redundant as well as distinct roles in neural development.

p35 knockouts also have defects in the bundling of nerve fibres of several prominent axon tracts [44]. Both Cdk5 and p35 are present in axon shafts and growth cones [45], and inhibition of Cdk5 activity in cultured neurons shows a decreased capacity to grow or main-

tain neuritic projections [45], as well as response to laminin [46]. In *Drosophila*, an increase or decrease of Cdk5 activity results in defects in axon pathfinding and target recognition of the abdominal motor nerves [47], further establishing a role for Cdk5 in axon guidance and targeting.

6. Deregulation of Cdk5 and neurodegeneration

There is growing evidence that Cdk5 kinase activity might be elevated during neurodegeneration and deregulation of Cdk5 activity is detrimental to cell survival [48,49]. When Cdk5 was initially isolated from mammalian brains, it was shown to exist in a heterodimeric complex with a 25 kDa regulatory protein (p25), which was subsequently demonstrated to be a truncated form of p35 [27,28,50]. The production of p25 (p29 in p39) in neurons correlates with the exposure to the agents that disrupt calcium homeostasis [51, 52]. Calpain, a calcium-dependent protease, induces the conversion of p35 to p25 [51,53]. Though p25 contains all the elements necessary for Cdk5 binding and activation [34,35], its *in vivo* properties are distinct from those of p35 [49]. Firstly, p25 has a substantially longer half-life than p35 and is able to associate with Cdk5 leading to a sustained activation of the kinase [49, 52]. Secondly, in brain extracts Cdk5-p35 exists in macromolecular complexes displaying little kinase activity while Cdk5-p25 exists as a highly kinase-active heterodimer. Thirdly, in contrast to p35, p25 lacks the myristoylation sequence for membrane targeting and therefore is not associated with the plasma membrane [49,52]. Presumably, the conversion of p35 to p25 relocates Cdk5 from its normal compartments since p25 is concentrated in the cell body and nucleus [49] (Fig. 2).

Both p35 and p39 contain several Cdk5 phosphorylation consensus sites. Phosphorylation of p35 by Cdk5 reduces its stability, leading to its degradation by the proteasome system, and hence prevents the cleavage of p35 by calpain [54]. Proteasome inhibition may thus keep the p35 level high. As such, the generation of p25 disrupts the normal regulation of Cdk5 by causing prolonged activation and mislocalization of Cdk5. One of the major pathological targets of the Cdk5/p25 complex is the microtubule-associated protein tau. Abnormal phosphorylation of tau has been implicated in the pathology of several neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases. The expression of p25/Cdk5 in cultured cortical neurons induced hyperphosphorylation of tau, neurite retraction, cytoskeletal abnormalities and apoptosis [49]. This is further supported by recent studies showing that mice overexpressing p25 caused hyperphosphorylation of tau and neurofilaments, cytoskeletal disruption and behavioral deficits reminiscent of AD [55,56]. Tau aggregation is a common trait of AD and hyperphosphorylation of tau has been implicated as an essential pathogenic means of this process. Mice overexpressing p25 display enhanced Cdk5 activity, and p25/tau double transgenic mice exhibit increased tau hyperphosphorylation and accumulation of highly aggregated tau in the brainstem and cortex, leading to the development of neurofibrillary tangles [56], which is a characteristic of many neurodegenerative diseases including AD. Therefore, Cdk5 may have a major impact on tau pathology progression, and hence, neurodegeneration.

7. Concluding remarks

Cdks are protein switches of the cell cycle that are commonly known to regulate cell proliferation. Interestingly, these typical cell cycle regulators can also affect apoptotic signaling, though no clear-cut pro- and anti-apoptotic effects can be described. Unlike classical Cdks, Cdk5 has no known role in the cell division cycle, but plays important regulatory functions in mammalian brain development. Growing evidence indicates that these cell cycle regulators, as well as Cdk5, are becoming mandatory components of the cell death pathways in neurons. Alteration in expression, cellular distribution and enzyme activity of these molecules have been observed in neurons of postmortem samples in AD, ALS, and several other neurodegenerative diseases. Moreover, aberrant activation of these Cdks can be triggered in brain neurons after cerebral ischemia or kainite-induced excitotoxicity as well as in cultured neurons after treatment with DNA-damaging agents, deprivation of toxic factors or β-amyloid peptide. Under such *in vivo* and *in vitro* conditions, neuronal cell death can be rescued by the use of CKIs or dominant negative forms of the kinases. Interestingly, CKIs have no effect on Cdk5, though a few protein inhibitors of Cdk5 have been identified. (unpublished observation) Although Cdk5 is known to be quite different from other Cdks, there are some interesting similarities between Cdk5 and its close relative, the mitotic kinase Cdc2 (Cdk1). For example, a similar process that might be regulated by Cdk5 in neurons and Cdc2 in dividing cells is the regulation of cytoskeleton-membrane cou-

pling events [57]. Furthermore, both kinases have identical substrate specificities *in vitro* and several proteins that function in both mitosis and migration are reported to be substrates of both kinases. Hence, it is desirable to develop kinase inhibitors that are highly specific for each subset of Cdks. More importantly, manipulating these Cdk activities may thus be beneficial for the treatment of those neurodegenerative diseases, an emerging killer in the ageing population.

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Review

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Protein-Protein Interactions in Cdk5 Regulation and Function

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Key Words

Cdk5 · Cdk5 activator · Cdk5 inhibitor · Cdk5 substrate phosphorylation · Protein-protein interaction, Cdk5 regulation/function

Abstract

Cdk5 is a unique member of the cyclin-dependent kinase (Cdk) family of small protein kinases. In association with its neuron-specific activator p35 or p39, Cdk5 displays many regulatory properties distinct from other Cdks. A growing body of evidence has suggested that Cdk5-p35 has important implications in a variety of neuronal activities occurring in the central nervous system. In brain, Cdk5-p35 appears to exist as large molecular complexes with other proteins, and protein-protein interactions appear to be a molecular principle for Cdk5-p35 to conduct its physiological functions. Over the past decade, a number of proteins have been identified to associate with Cdk5-p35. While the majority of these proteins mediate their interaction with Cdk5 through p35, implying that p35 may act not only as an activator of Cdk5 but also as an adaptor to associate Cdk5 with its regulators and physiological targets, a small group of other proteins are found to link directly with Cdk5. In addition, Cdk5 has been found to phosphorylate a diverse list of substrates, further implicating its regulatory roles in a wide range of cellular processes. In this review, we present an updated inventory of the interacting proteins of Cdk5-p35 kinase and its substrates as well as a discussion on the implicated effects of these interactions.

Introduction

The process by which extracellular signals are relayed from the plasma membrane to specific intracellular sites is highly regulated to control and alter many cellular functions. Protein phosphorylation is one such cellular mechanism, and protein kinases contribute in a variety of ways to mammalian signal transduction pathways [1, 2]. Cyclin-dependent kinases (Cdks), with the exception of Cdk3 and Cdk5, require the binding of cyclin for their activation [3, 4]. The active Cdk enzymes mediate the cell cycle progression by phosphorylating a variety of protein substrates at the proline-directed Ser/Thr residues. Moreover, the definition of Cdks does not limit their biological functions to the control of cell proliferation. They also play a role in differentiation, senescence, and apoptosis [5].

Cdk5 was initially identified independently by virtue of its close sequence homology to human Cdk1 (Cdc2), by biochemical purification from bovine brain based on its

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proline-directed Ser/Thr kinase activity, and by affinity isolation as a cyclin D1-associated protein in fibroblasts [6–8]. Cdk5 is an atypical member of the Cdk family. Despite its close sequence homology with Cdk1, it is not activated by any cyclin, although it can bind cyclin D1 and cyclin E [8, 9]. The first known activators of Cdk5 are p35 and its proteolytic product p25 which were isolated as binding partners of Cdk5 in the brain extract [10]. p25, which is a 208-residue carboxyl-terminal fragment of p35, retains the Cdk5 binding and activating domain of p35 [11, 12]. Another activator of Cdk5, p39, was identified by its sequence homology to p35, with which it shares 57% amino acid identity [13, 14]. Monomeric Cdk5 does not display any enzymatic activity. The binding to p35, p25, or p39 activates its kinase activity in the absence of any Cdk5 modification and association of any other protein factors [10, 15, 16]. Though Cdk5 is a ubiquitously expressed protein, its kinase activity is restricted to the nervous system by the neuron-specific expression of its activators p35 and p39 [17].

Although Cdk5 is a member of the Cdk family, it is not involved in cell cycle regulation. Since its discovery more than a decade ago, Cdk5 has been shown to play an important role in many cellular processes occurring within neurons in the central nervous system (CNS) [18]. For example, Cdk5 is well known to participate in the regulation of cytoskeleton organization, axon guidance, membrane transport, synaptic function, dopamine signaling, and drug addiction [19]. Gene-targeting experiments have demonstrated an essential role of Cdk5 in the cellular organization of the CNS. Mice that are deficient of *Cdk5* die just before or after birth and show widespread disruptions in the neuronal layering of many brain structures [20–22]. The lethality of the *Cdk5*-deficient mice is likely to be a result of the defects in the nervous system, since it can be completely rescued by expressing the *Cdk5* transgene under the p35 promoter [23]. In contrast to *Cdk5*-deficient mice, $p35^{-/-}$ mice are viable and fertile, though they have an increased susceptibility to seizures [24, 25]. *p35*-deficient mice show an inverted layering of cortical neurons comparable to that observed in the $Cdk5^{-/-}$ mice, but have only mild disruptions in the hippocampus and have a fairly normal cerebellum. *p39/p35* double knockout mice display the same phenotype as the $Cdk5^{-/-}$ mice, further establishing these proteins as the primary activators of Cdk5 [17].

It has been shown that cellular Cdk5 exists in three forms: free monomeric Cdk5, a heterodimeric complex of Cdk5-p25, and multiprotein complexes of Cdk5-p35 [26, 27]. As revealed by a protein fractionation procedure, Cdk5-p35 exists as large molecular complexes of more than 670 kD in the extract of brain tissues. Consistently, an increasing number of proteins has been reported to associate with Cdk5-p35, linking Cdk5 to its physiological functions. This article discusses the regulatory and functional properties of Cdk5 in relation to its known interacting proteins and substrates.

Molecular Organization of Cdk5 Complexes

A body of evidence has suggested that Cdk5-p35 shows a high-affinity binding to specific cellular proteins. To date and to our best knowledge, there are about 30 proteins with diverse functions being identified to associate to Cdk5-p35 or Cdk5-p39 (table 1). Cdk5 appears to bind directly with a small subset of these proteins which include Cables, tau, PP1, dbpA, and L34. Many of the other proteins interact with Cdk5 via p35 or p39, implicating that p35 and p39 not only act as the activators of Cdk5 but also are important mediators of the Cdk5 functions. In addition, a number of proteins have been established to be substrates of Cdk5 (table 2). The majority of these substrates, as well as the Cdk5-p35- and Cdk5-p39-associated proteins, have revealed numerous important functional and regulatory properties of Cdk5.

Cdk5, p35, and p39 are abundantly expressed in adult brains, and high levels of Cdk5 kinase activity are detected in postmitotic neurons of the nervous system, in accordance with the expression pattern of p35 and p39. As neurons differentiate, cell cycle Cdks are downregulated, while the Cdk5 activity is increased [11]. p35 is highly expressed in the postmitotic neurons of the developing cortex, but is not found in proliferating neuronal precursors. On the other hand, the highest level of p39 expression in the CNS occurs postnatally. Apparently, p35 and p39 display an overlapping, but distinct temporal and spatial pattern in brain [28]. Thus, Cdk5-p39 may arbitrate functions distinct from those involving Cdk5-p35 during neurodevelopment.

Using immunocytochemistry and cellular fractionation protocols, Cdk5 and p35 proteins were detected throughout the cells with a much lower level in the nucleus [29]. p35 is enriched in the membrane fraction, and the association of Cdk5-p35 with the plasma membrane is directed by the myristoyl moiety linked to the N-terminal glycine of p35 [30, 31]. Moreover, Cdk5-p35 extracted from a membrane preparation of rat brains exhibited the biochemical property of large molecular complexes [Gao and Qi, unpubl. observation]. Conceiv-

Table 1. Proteins associated with Cdk5 and p35

Associated protein	Putative function	Reference No.
Cables	enhances c-Abl's association and phosphorylation of Cdk5 at Tyr15	47
Fyn	promotes Cdk5 activity through phosphorylation of Tyr15	48
SET	enhances Cdk5-p35 activity	29
C42	inhibits Cdk5 activation by p35	50
Ribosomal protein L34	inhibits Cdk5-p35 activity	53
DbpA	inhibits Cdk5-p35 activity	52
Ubiquitin	proteasomal degradation of p35	85
Rac	phosphorylation of PAK1	55, 86
PAK1	Phosphorylation of PAK1	55, 86
Actin	potential role in actin dynamics	14
α-actinin1	potential role in the localization of Cdk5 to the synaptic cytoskeleton	70
NFH/NFM	phosphorylation of NFH/NFM	10, 58
Tau	phosphorylation of tau	16, 87
Munc 18	phosphorylation of Munc 18	65, 68
Syntaxin 1A	mediates trafficking and secretion	68
Amphiphysin 1	phosphorylation of amphiphysin 1	67
CaMKIIα	regulation of synaptic plasticity	70
β-Catenin	regulates N-cadherin-mediated cell adhesion and the association of β-catenin with presenilin 1	71, 72
PP1	phosphorylation of PP1.I-2	88
pRb	phosphorylation of pRb	79
PCTAIRE1	phosphorylation of PCTAIRE1	36
Lipofuscin	putative pathogenic process of ALS	89
ErbB	mediates neuregulin-induced AChR expression at neuromuscular junction	90
C48	not known	82
C53	not known	82
IC53	human homologue of rat C53 and potential role in cell proliferation	91
IC53-2	human homologue of rat C53 and potential role in cell proliferation	92
Cyclin D	Cdk5 binding protein and potential regulator of Cdk5	8
Cyclin E	Cdk5-binding protein and potential regulator of Cdk5	9
Ik3-2	homologue of Cables (ik3-1)	93

Table 2. Cdk5 substrates

Cdk5 substrate	Putative function of phosphorylation	Reference No.
p35	suppresses the proteolytic conversion of p35 to p25 by calpain and facilitates the proteasomal degradation of p35	85
Cables	mediates its interaction with Cdk5	47
PAK1	inhibits PAK1 activity and regulates cytoskeletal dynamics	55, 86
NFH/NFM	regulates axonal transport of neuro-filaments	58, 94
Tau	regulates microtubule binding and dynamics	87
MAP1B	regulates microtubule stability	95, 96
Nudel	regulates dynein-mediated axonal transport	64
Synapsin 1	regulates synaptic transmission	66
Munc 18	mediates the interaction between Munc 18 and syntaxin 1A	68
Amphiphysin 1	regulates synaptic vesicle endocytosis and neurite outgrowth	67
Dynamin 1	facilitates synaptic vesicle endocytosis	69
NR2A (N-methyl-*D*-aspartate receptor)	regulates synaptic transmission and plasticity	78
Src	regulates functions of Src in neurons	97
β-APP	mediates APP localization and function	98
β-catenin	regulates N-cadherin-mediated cell adhesion and the association of β-catenin with presenilin 1	71, 72
Presenilin 1	regulates PS1 stability and metabolism	99
P/Q type calcium channel	inhibits the neurotransmitter release in synaptic transmission	77
DARPP-32	regulates dopamine signaling and the stimulant action of caffeine	73, 100
MEK1	inhibition of MEK1 activity	76
JNK3	inhibits JNK3 activity and mediates neuronal apoptosis	75
PP1 inhibitors I-1/I-2	activates I-1 and I-2 to mediate PP1 activity	88, 101
pRb	mediates neuronal apoptosis	79
p53	mediates p53 transcriptional activity	80
MEF2	inhibits MEF2 transcriptional activity and mediates neuronal apoptosis	81
Dab1	mediates neuronal migration	102
PCTAIRE1	promotes the PCTAIRE1 activation	36
ErbB	mediates neuregulin signaling at the neuromuscular junction	90
P_γ (retinal cGMP phospho-diesterase)	mediates PDE activity and phototransduction	103, 104

ably, the membrane localization is important for Cdk5 to exert many of its physiological effects, and some of its substrates are likely to be membrane-integral or membrane-associated proteins. Additionally, an active Cdk5-p35 kinase has been shown to be present in Golgi membranes, where it associates with a detergent-insoluble fraction containing actin [32]. Suppression of the Cdk5 activity blocks the formation of membrane vesicles from the Golgi apparatus, possibly suggesting a role for Cdk5-p35 in membrane trafficking. Furthermore, Cdk5-p35 may be involved in the reorganization of actin in the growth cone and on the Golgi membrane during neurite outgrowth.

Under certain conditions, p35 is converted to p25 by proteolytic cleavage, losing the smaller N-terminal fragment of p35, commonly known as p10 [33, 34]. The transformation of p35 to p25 appears to lose most of the components of the Cdk5-p35 macromolecular complexes, implicating that the p10 region of p35 might be required for interaction with these proteins. Indeed, deletion analyses have provided proof of specific and direct interaction between p10 and at least one p35-interacting protein [29]. Another region of 26 amino acids, spanning residues 145–170 of p35, has also been identified to contain the binding site for a few of the identified p35-binding proteins [35, 36]. This short stretch is proximal to the N-terminal boundary of the Cdk5-binding and Cdk5-activating domain in p35 and contains an amphipathic α-helix [37]. Further evidence from interaction studies indicates that the hydrophilic face of the helix is involved in the interaction with the binding proteins, while the hydrophobic face is involved in the association with Cdk5 [35]. Possibly this unique feature of the p35 structure is necessary to support a number of other functions when bound to its interacting proteins, in addition to the kinase activation.

Cdk5 Regulation Imposed by Regulatory Proteins

The association with a cyclin is essential in the activation of Cdks. However, the Cdk5 activity has not been found to associate with any cyclin. Instead, p35 and p39 were found to be two specific activators of Cdk5. Although p35 and p39 have little sequence similarity to any cyclin, studies by computer modeling and mutagenesis have predicted that p35 might adopt a cyclin-like tertiary structure [38–40]. Recently, these predictions were further established by results from crystallization of a Cdk5-p25 complex [41].

Members of the Cdk family are also regulated by at least three distinct phosphorylation/dephosphorylation events. Phosphorylation of Cdk1 and Cdk2 at Thr14 and Tyr15 by the dual-specificity kinases Wee1, Myt1, and Mik1 inhibits their activities [42–44]. In contrast, phosphorylation of Thr160 in the T-loop of Cdk2 (or Thr161 of Cdk1) by the Cdk-activating kinase is necessary for its maximal activation [45]. Though Thr14 and Tyr15 are conserved, and Thr160 in Cdk2 is conservatively substituted with Ser159 in Cdk5, and their surrounding sequences are highly homologous to those of the authentic Cdks, Cdk5 appears to adopt regulatory mechanisms distinct from those of the classical Cdks at these three phosphorylation sites. The Thr14 and Tyr15 sites in Cdk5 are not phosphorylated by Wee1 in vitro [46]. Moreover, Tyr15 of Cdk5 can be phosphorylated by the cytosolic tyrosine kinase c-Abl, and such phosphorylation is facilitated by the Cdk5 association with Cables which is an Abl-binding protein. Surprisingly, the phosphorylation of Cdk5 at Tyr15 is stimulatory and enhances the Cdk5 kinase activity [47]. In addition to c-Abl, Fyn, which is a member of the Src family of tyrosine kinases, is the other enzyme observed to catalyze the stimulatory Tyr15 phosphorylation of Cdk5 [48]. The Cdk5 phosphorylation by Fyn is necessary for the semaphorin-3A-induced growth cone collapse in model neurons [48]. Lastly, the phosphorylation of Cdk5 at Ser159, which occupies a position equivalent to the Thr160 site in the conserved T-loop of Cdk2 (Thr161 of Cdk1), not only is dispensable, but also dampens the activation of Cdk5 [41]. The crystal structure of Cdk5-p25 revealed that the interaction between the regulatory subunit is sufficient to stretch the activation loop of unphosphorylated Cdk5 into a fully extended active conformation, analogous with the phosphorylated Cdk2-cyclin A complex [41].

Another mode of the Cdk regulation involves a diverse family of inhibitory proteins (CKIs) that bind Cdks or Cdk-cyclin complexes to inhibit the Cdk activity [5]. The initial evidence of the existence of Cdk5 inhibitors came from the biochemical separation of Cdk5 complexes in brain extract. The Cdk5-p35 macromolecular complexes are neither enzymatically active nor activable by the addition of a truncated form of p35 [26]. Furthermore, the kinase activity was recovered, when the Cdk5-p35 complexes were further fractionated by the size-exclusion chromatography in the presence of 10% ethylene glycol, suggesting that an inhibitor(s) could be dissociated from the complexes under this stringent condition. Interestingly, Cdk5 is not regulated by any of the CKIs that are known for other Cdks, such as members of the INK and

CIP/KIP families of inhibitors [49], suggesting distinct structural and regulatory properties of Cdk5-p35. A few protein candidates have been reported to be physiological inhibitors of Cdk5. C42, which is a p35-binding protein, has been shown to specifically inhibit the activation of Cdk5 by p35 [50]. The inhibitory domain of C42 was mapped to a region of 135 amino acids which is conserved in Pho81, a yeast protein that inhibits the yeast cyclin-dependent protein kinase Pho85 (yeast functional homologue of mammalian Cdk5) [51]. DNA-binding protein dbpA and ribosomal protein L34 are two other reported inhibitors of Cdk5 [52, 53]. They were identified in a yeast two-hybrid screen as Cdk5-binding proteins. In addition to the inhibitors, the nuclear protein SET was found to enhance the Cdk5 activity upon its physical association with Cdk5-p35. The SET protein binds p35 in its N-terminal region, which is lacking in p25, and, therefore, does not affect the activity of Cdk5-p25, suggesting specific modulation of the Cdk5-p35 activity in the nucleus [29].

Targets of Cdk5 and Mediated Functions

Over the last decade, a number of proteins have been identified to act as direct substrates of Cdk5 (table 2), providing a good deal of knowledge on the biological roles of Cdk5 in brain. It appears that Cdk5 acts prominently in many of the essential cellular processes, including cytoskeletal dynamics, cell adhesion, axonal guidance, dopaminergic signaling, and synaptic membrane functions.

Cdk5 in Cytoskeletal Dynamics and Microtubule-Based Transport

A body of evidence has implicated an indispensable role of the Cdk5-p35 kinase in axonal guidance, cell motility, and neurite outgrowth. Overexpression of p35 in cultured neurons induces the formation of longer neurites, whereas inhibition of the Cdk5 activity or the expression of a dominant negative form of the kinase prevents neurite outgrowth [54]. Cdk5-p35 colocalizes with F-actin, Rac, and PAK1 on the periphery of growth cones. Since Cdk5-p35 downregulates the PAK1 kinase activity by phosphorylating it at Thr212, it has been proposed to have a role in regulating actin repolymerization and, therefore, growth cone dynamics [55].

Another group of Cdk5 substrates are the intermediate and heavy chains of neurofilament proteins (NFM and NFH, respectively). NFM and NFH contain many KSP (Lys-Ser-Pro) repeats in their long carboxyl terminal tails. Phosphorylation at the KSP sites occurs during axonal transport of neurofilaments, and phosphorylation is required for maintaining the axonal morphology. Cdk5 was originally isolated from the brain as a neurofilament kinase to catalyze the KSP phosphorylation at the tail region of NFM and NFH [56]. Phosphorylation of these domains by Cdk5 reduces their association with microtubules as well as retards the axonal transport of these proteins [57]. In vitro, NFH phosphorylated by Cdk5 displayed the same electrophoretic motility shift as that of natively phosphorylated NFH [58]. Moreover, p35 associates with NFM and NFH in a region adjacent to the KSP-rich domains, suggesting a role of p35 in docking Cdk5 to the substrates [59].

It has been found that Cdk5 associates with microtubules and that it can be copurified with microtubules from bovine brain [60, 61]. Several microtubule-associated proteins, such as tau and MAP1B, are substrates of Cdk5. Phosphorylation of tau by Cdk5 abolishes the ability of tau to bind microtubules and, therefore, its ability to promote microtubule assembly [57]. In addition to phosphorylation of the microtubule-associated proteins to mediate microtubule stability, Cdk5 has been implicated to play a role in regulating the dynein-mediated axonal transport. Nudel is a cytoplasmic dynein-associated protein that is expressed at a high level in the brain. Cdk5 can phosphorylate Nudel in vitro and in vivo, and this is of importance, since the introduction of a nonphosphorylatable mutant of Nudel into the cultured neurons led to axonal swelling, analogous to disruption of the dynein function in *Drosophila* neurons [62].

Cdk5 in Synapses and Focal Adhesion Sites

Cdk5, p35, and p39 are present in subcellular fractions enriched for synaptic membrane, and they are localized to the pre- and postsynaptic compartments [63, 64], indicating that they may be involved in synaptic functions. Indeed, several synaptic proteins have been identified as Cdk5 substrates, including Munc 18, synapsin I, and amphiphysin which are proteins implicated in synaptic vesicle exocytosis [65–67]. Phosphorylation of Munc 18 by Cdk5 results in disassembly of the Munc 18-synaxin I complex, implying a role for Cdk5 in modulating neurosecretion [68]. Most recently, interesting findings by Tan et al. [69] established that Cdk5 has a role in synaptic vesicle endocytosis. Cdk5 phosphorylates dynamin I in vitro as well as in vivo at the nerve terminals of neuronal cells to facilitate synaptic endocytosis [69]. Roscovitine, an antagonist of the Cdk5 activity, blocks the rephosphorylation of dynamin I after repolarization of the synaptosomes. Furthermore, phosphorylation by Cdk5 also in-

creases the GTPase activity of dynamin I [69]. Hence, Cdk5 may play a major role at synapses, since multiple Cdk5 substrates are involved in the synaptic vesicle recycling. In a yeast two-hybrid screen, α-actinin 1 and the α-subunit of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKIIα) were identified as p35- and p39-interacting proteins [70]. Either of these two proteins forms a complex with Cdk5 through the interaction with p35 or p39, and these interactions potentially localize Cdk5 to the postsynaptic density, where it may play a role contributing to synaptic plasticity, memory, and learning.

N-cadherin is a member of the transmembrane molecules that promote cell adhesion by their calcium-dependent homophilic interactions. The cytoplasmic tail of cadherins interacts with α- and β-catenin to anchor the cadherins to the actin cytoskeleton. Cdk5-p35 is associated with β-catenin and controls the N-cadherin/β-catenin-mediated cell adhesion through the phosphorylation of β-catenin [71, 72]. Additionally, phosphorylation of β-catenin by Cdk5 has also been shown to affect its binding to presenilin which has a role in Alzheimer's disease pathology [72].

Cdk5 in Neurosignaling

Accumulating evidence has implicated that Cdk5 is involved in many of the neuronal signal transduction pathways. DARPP32 is a Cdk5 substrate that plays a key role in dopamine signaling occurring in the dopaminoceptive neurons. When DARPP32 is phosphorylated by the cAMP-dependent protein kinase (PKA), it becomes a potent inhibitor of protein phosphatase 1. It appears that the Cdk5 phosphorylation of DARPP32 transforms it into an inhibitor of PKA, exerting an opposing effect on dopamine signaling [73, 74]. In the MAPK (mitogen-activated protein kinase) and JNK (c-Jun N-terminal kinase) pathways, Cdk5 is associated with JNK-3, where it inhibits the JNK-3 activity by phosphorylating JNK-3 at Thr131 to mediate neuronal apoptosis [75]. Meanwhile, Cdk5 downregulates the MAPK signaling by phosphorylating MEK1 at Thr286 and, therefore, inhibiting its activity [76]. In addition, Cdk5 has been shown to mediate the activities of the P/Q-type voltage-dependent calcium channel and the N-methyl-*D*-aspartate class of glutamate receptors through a direct phosphorylation modification [77, 78].

Cdk5 in Transcriptional Machineries

Recent studies have pointed to the localization of Cdk5-p35 in the nucleus, where it may play a role in transcriptional regulation. In an earlier report [79], Cdk5 was found to bind and to phosphorylate the retinoblastoma protein (pRb). p53 is a phosphorylation-regulated transcription factor that plays a pivotal role in cell cycle progression and cell death. Cdk5 is able to modulate the p53 transcriptional activity through direct phosphorylation of p53 as well as elevation of the p53 expression in the cells [80]. More recently, myocyte enhancer factor 2 (MEF2) was identified as a Cdk5 substrate [81]. Members of the MEF2 family are transcription factors that play critical roles in diverse cellular processes, including neuronal survival. Phosphorylation of MEF2 by Cdk5 results in the inhibition of the MEF2 transactivation activity, while MEF2 mutants that are resistant to the Cdk5 phosphorylation rescue neurons from neurotoxin/Cdk5-induced apoptosis, suggesting the MEF2 phosphorylation by Cdk5 as a part of the molecular mechanism by which neurotoxin/Cdk5 mediates apoptosis [81].

Methods Used in Identifying Cdk5 Complexes

Various methods have been described in an increasing number of reports over the last few years to uncover the protein components of Cdk5 macromolecular complexes in the brain. Using the yeast two-hybrid system, a number of proteins have been found by screening mammalian brain libraries to specifically interact with Cdk5, p35, or p39 [52, 70, 82]. This is a sensitive method with which transient or weak interactions can be detected. However, it could be disadvantageous for the screening of membrane-associated proteins and proteins which are not able to translocate into the nucleus. The usage of this method is also limited to the detection of dimeric protein complexes and, therefore, is disadvantageous for identifying multimolecular complexes. Further, some interactions may require certain posttranslational modifications of their protein partners. However, such modifications may not occur in yeast.

Another major method to identify protein-protein interactions at a high throughput and at a proteome-wide scale is biochemical isolation of the protein complexes from animal tissues or cultured cells [83, 84]. In our laboratory, biochemical isolation of Cdk5 and p35-associated proteins was conducted using affinity chromatography media which were prepared by coupling antibodies recognizing Cdk5 or p35 or by coupling recombinant proteins derived from Cdk5 and p35 to agarose beads [29]. The identity of isolated interacting proteins from rat brain extracts was established using mass spectrometry. An advantage of this method is the ability to isolate protein

complexes and interacting proteins in their native states. It is especially useful in the identification of indirect interacting proteins, since multiprotein complexes can be isolated. However, it is less favorable with proteins having weak or transient interactions. Therefore, this approach would be a good complement to the yeast two-hybrid methodology.

Concluding Remarks

A variety of cellular proteins have been found that undergo specific and high-affinity association with Cdk5-p35 or Cdk5-p39. Some of these protein-protein interactions have been further characterized, and this characterization has provided insights into regulation, function, and mechanism of the actions of Cdk5-p35 and Cdk5-p39. Although Cdk5 was identified as a member of the Cdks, it is not activated by any cyclin protein, nor is it involved in regulating cell cycle progression. In contrast, it is involved in various activities in postmitotic neurons, including modulating the neuronal migration in the developing CNS. Cdk5 has also been implicated in pathological pathways of several neurodegenerative diseases. Thus, the identification of Cdk5-associated proteins and Cdk5 substrates at the proteome-wide scale will lead us towards a complete understanding of the role of Cdk5 in the nervous system. With the availability of the human genome sequences, building a functional proteome on Cdk5 will further elucidate many more exciting insights into the molecular basis of neurodevelopment and progression of diseases.

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Direct Regulation of Microtubule Dynamics by Protein Kinase CK2*

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Microtubule dynamics is essential for many vital cellular processes such as morphogenesis and motility. Protein kinase CK2 is a ubiquitous protein kinase that is involved in diverse cellular functions. CK2 holoenzyme is composed of two catalytic α or α' subunits and two regulatory β subunits. We show that the α subunit of CK2 binds directly to both microtubules and tubulin heterodimers. CK2 holoenzyme but neither of its individual subunits exhibited a potent effect of inducing microtubule assembly and bundling. Moreover, the polymerized microtubules were strongly stabilized by CK2 against cold-induced depolymerization. Interestingly, the kinase activity of CK2 is not required for its microtubule-assembling and stabilizing function because a kinase-inactive mutant of CK2 displayed the same microtubule-assembling activity as the wild-type protein. Knockdown of CK2α/α' in cultured cells by RNA interference dramatically destabilized their microtubule networks, and the destabilized microtubules were readily destructed by colchicine at a very low concentration. Further, over-expression of chicken CK2α or its kinase-inactive mutant in the endogenous CK2α/α'-depleted cells fully restored the microtubule resistance to the low dose of colchicine. Taken together, CK2 is a microtubule-associated protein that confers microtubule stability in a phosphorylation-independent manner.

Protein kinase CK2 (formerly known as casein kinase 2) is ubiquitously expressed and highly conserved in eukaryotic cells (1–4). It comprises two catalytic α or α' subunits and two regulatory β subunits to form a heterotetrameric structure in which the two β subunits dimerize to link the two α or α' subunits (5). As a protein serine/threonine kinase, CK2 has a very broad phosphorylation spectrum, and over 300 protein substrates of CK2 have been identified to date (6). A number of studies have indicated that CK2 is involved in a wide variety of cellular processes including cell cycle, apoptosis, transcriptional regulation, and signal transduction (1, 3, 6). CK2 is instrumental and necessary for promoting cell survival (3, 7). Disruption of genes encoding both of the catalytic subunits of CK2 is synthetic lethal in fission yeast (8, 9). Similarly, it is embryonic lethal when CK2β is knocked down in *Caenorhabditis elegans* by RNA interference or in mice by gene disruption, reminiscent of an essential role of CK2β during embryonic development and organogenesis (10, 11). Hence, production of both the α and β subunits of CK2 appears to be mandatory for cell viability.

A few lines of evidence have lead to implication that CK2 might be involved in the regulation of microtubule cytoskeleton reorganization (12–14). CK2 was localized to microtubule structures such as the mitotic spindle of dividing cells and was found to associate with the cold-stable fraction of microtubules from the rat brain (14, 15). More recently, the α and α' subunits were shown to bind tubulin in a far Western assay (16). Further, CK2 is able to phosphorylate a number of microtubule elements, including MAP1B and a neuron-specific β-tubulin isotype (6). The phosphorylation of MAP1B was proposed to facilitate the microtubule association of MAP1B and thereby microtubule assembly, whereas the physiological role of the β-tubulin isotype phosphorylation is still unclear (12, 17). Despite these findings, the direct correlation of CK2 and microtubule stability has not been established.

In the present study, we have investigated the physical association of CK2 with microtubules and the direct effect of CK2 on microtubule dynamics. Our results show that CK2 is a microtubule-associated protein (MAP)[1] that induces microtubule assembly and bundling *in vitro*. CK2-polymerized microtubules appear stable under cold treatment. In cultured cells, knockdown of CK2α/α' has a severe effect on microtubule stability, which implies that CK2 mediates microtubule integrity *in vivo*. Moreover, a kinase-inactive mutant of CK2 displayed the same microtubule polymerizing and stabilizing activity *in vitro* and *in vivo*. Thus, the microtubule assembling and stabilizing action of CK2 is independent of its kinase function.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The coding sequences of chicken CK2α and its kinase-inactive mutant (CK2αK68A) were subcloned into pGEX4T (Amersham Biosciences), pET32 (Novagen), and pDneo-Myc (18). The full-length sequence of human CK2β was cloned by a reverse transcription polymerase chain reaction and inserted into pQE30 (Qiagen).

Protein Binding Assay—Proteins tagged with GST or His_6 were bacterially expressed and prepared as described previously (19). To test tubulin binding, GSH-Sepharose beads (Amersham Biosciences) prebound with GST, GST-CK2α, or the complex of GST-CK2α/His-CK2β were incubated with purified tubulin (>99% pure and MAP-free, Cytoskeleton) for 1 h at 4 °C. After being extensively washed with binding buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 20 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1% Nonidet P-40), the beads were boiled in SDS-PAGE sample buffer and analyzed by immunoblotting. Antibodies against α- and β-tubulin were from Sigma. The binding of His-tagged proteins with tubulin was performed with nickel-nitrilotriacetic acid beads (Ni-NTA, Qiagen) in binding buffer without dithiothreitol. In the microtubule binding assay, microtubules, which were pre-assembled using taxol in PEM buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) supplemented with 1 mM GTP, were incubated with the indicated proteins. The samples were subsequently loaded onto a buffered cushion (50% glycerol in PEM buffer) and centrifuged to spin down the

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[1] The abbreviations used are: MAP, microtubule-associated protein; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; siRNA, small-interfering RNA; PIPES, 1,4-piperazinediethanesulfonic acid.

microtubules and associated proteins. The pellet and the supernatant were analyzed by immunoblotting.

Microtubule Assembly—Microtubules were assembled *in vitro* from the purified MAP-free tubulin at 2 mg/ml in PEM buffer supplemented with 1 mM GTP at 35 °C, and the turbidity of the solutions was monitored at 340 nm (20). CK2 was added at various amounts as indicated to promote the assembly. To visualize assembled microtubules, tubulin and rhodamine-labeled tubulin (Cytoskeleton) at the ratio of 7:1 were used in the polymerization (21). Microtubules were fixed with 0.5% gluteraldehyde and visualized by fluorescence microscopy.

Differential Tubulin Extraction from Intact Cells—Differential extraction of tubulin heterodimers and polymers from cells was performed using a protocol described previously (22). Briefly, cultured cells were lysed with the microtubule-stabilizing buffer (80 mM PIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 10% glycerol, and Roche protease inhibitor mixture), which was prewarmed to 35 °C, to extract cytosolic soluble tubulin heterodimers and preserve microtubules (assembled insoluble tubulin polymers). The extract was cleared by centrifugation and the supernatant designated as the free tubulin fraction. After a brief washing with the microtubule-stabilizing buffer, the pellet was extracted in the microtubule-destabilizing buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 mM $CaCl_2$, and Roche protease inhibitor mixture). The extract was clarified by centrifugation to yield the polymerized tubulin fraction. Both fractions were analyzed by immunoblotting, and each band on the blots was quantitated using a Bio-Rad GS-700 imaging densitometer and analyzed with the Multi-Analyst, version 1.0.1, program (Bio-Rad).

Cell Culture, Transfection, and Immunofluorescence—COS-7, HeLa and 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The siRNA sequence designed for human CK2α/α' is 5′-CCAGCUGGUAGUCAUCUUGUU-3′, which has a few discrepancies with the corresponding sequence of chicken CK2α/α'. 20 μM CK2α/α' siRNA or a scrambled siRNA sequence was applied into the transfection using a *Trans*IT-TKO transfection reagent (Mirus). Simultaneous transfection of siRNA and plasmid DNA was done using *Trans*IT-TKO and LipofectAMINE (Invitrogen) concurrently. After transfection, the cells were cultured for 24 h before treatment with 0.2 μM colchicine (Sigma) for 3 h. The cells were subjected to differential extraction of free and polymerized tubulin or to immunostaining. For immunofluorescence, the cells were fixed in PBS containing 4% paraformaldehyde and then permeabilized in PBS containing 0.2% Triton X-100. After a blocking wash with 10% goat serum and 0.1% Triton X-100 in PBS, immunostaining was performed with antibodies as indicated. CK2α- and CK2β-specific antibodies were from Santa Cruz Biotechnology. The secondary antibodies are Fluor594 goat anti-mouse IgG and Fluor488 donkey anti-goat IgG (Molecular Probes). The cells were then washed in PBS, mounted, and photographed on an MRC-1024 laser scanning confocal microscope (Bio-Rad).

RESULTS

CK2 Forms a Direct Complex with Microtubules—The direct association of CK2 and microtubules was probed by a series of binding assays using recombinant CK2 and purified MAP-free tubulin as well as pre-assembled microtubules. The α/β heterodimer of tubulin was found to associate with the catalytic α subunit as well as the holoenzyme of CK2 (Fig. 1*A*). CK2β alone did not result in the pull-down of any tubulin (Fig. 1*B*), which is in agreement with a previous observation using far Western blotting (16). To verify the microtubule association of CK2, taxol-assembled microtubules were incubated with CK2 holoenzyme or its individual subunit proteins. The microtubules were then spun down to test whether these proteins co-precipitated with the microtubules. Consistently, CK2α and the holoenzyme of CK2 were found to associate with the microtubule pellet, whereas CK2β and GST, as a control protein, failed to co-precipitate with the microtubules (Fig. 1*C*), indicating that the CK2 holoenzyme associates with microtubules at a high affinity through CK2α.

Cellular localization of CK2 to microtubule networks was revealed by immunofluorescent staining of cultured COS-7 cells. Microscopic imaging of endogenous CK2α and CK2β displayed a clearly defined positioning with the microtubule network, particularly in the cell periphery (Fig. 2*A*). As confirmation, pools of tubulin existing as free heterodimers or polymers (microtubules) were differentially extracted from the cultured cells to examine the distribution of CK2 (22). Both CK2α and CK2β appeared in the microtubule fraction as well as the fraction of free tubulin heterodimers, although there appeared to be more CK2β in the microtubule fraction (Fig. 2*B*). Taken together with the results from the *in vitro* binding assays, this provides evidence of the direct association of CK2 with cellular microtubules.

CK2 Induces Microtubule Polymerization—We next investigated whether CK2 has any effect on microtubule dynamics by using an *in vitro* assay of microtubule assembly from purified MAP-free tubulin (20). During the assay, the turbidity change of the solution was measured as tubulin polymerizes or depolymerizes. In the absence of CK2, there was minimal polymerization of tubulin even after a prolonged incubation (Fig. 3, *A* and *B*). The addition of CK2 at a ratio of 1:240 to tubulin resulted in substantial polymerization of tubulin into microtubules (Fig. 3, *A* and *B*). Clearly, both the rate and extent of polymerization were dramatically enhanced by CK2. When the amount of CK2 was increased, tubulin polymerization was increased in a dose-dependent manner (Fig. 3, *A* and *B*). To verify the microtubule formation, rhodamine-labeled tubulin was applied into the polymerization experiments for direct visualization of the assembled microtubules by fluorescence microscopy (21). As shown in Fig. 3*C*, microtubule filaments and bundles were readily observed with the CK2-incubated tubulin, whereas the incubation of tubulin without CK2 showed no obvious microtubule formation. Therefore, we have found that CK2, in addition to showing high affinity binding to tubulin and microtubules, induces the assembly of tubulin into microtubules. Moreover, CK2 appeared to cause microtubule bundling, suggesting a strong stabilizing effect on the microtubules.

CK2 holoenzyme is a tetrameric complex of two α or α' subunits and two β subunits (5). Given that observation that CK2α of the holoenzyme interacts with microtubules, we explored whether the microtubule assembling function of CK2 is restricted to the holoenzyme by applying the α and β subunits of CK2 individually into the microtubule assembly assay. In contrast to the holoenzyme, when either the α or β subunit was tested, there was minimal polymerization of tubulin even after a prolonged incubation (Fig. 4). The CK2α- and CK2β-polymerized samples had no marked difference from the background tubulin polymerization, which was shown in the GST-incubated sample. Thus, only CK2 holoenzyme, but not each of the individual subunits, has the ability to induce microtubule assembly even though CK2α has shown microtubule binding activity.

CK2 has been known to catalyze phosphorylation of a neural isoform of β-tubulin and some of the MAPs, raising the possibility that it may affect microtubule dynamics through a kinase reaction (12, 17). Although ATP was not present in the *in vitro* microtubule assembly assay, CK2 is capable of utilizing either ATP or GTP as the phosphate donor in its phosphorylating reactions (23). We designed an experiment to assess the role of CK2 kinase activity in microtubule assembly. A kinase-inactive holoenzyme of CK2, in which CK2α was replaced with the kinase-inactive mutant CK2αK68A, was tested in the microtubule assembly assay. Fig. 5 shows that the kinase-inactive CK2 conferred the same microtubule polymerizing activity as the wild-type enzyme, indicating that the microtubule assembly entity of CK2 is independent of its kinase activity and phosphorylation of any microtubule proteins.

Microtubules from brains can be separated into two pools, namely "cold labile" and "cold stable," according to whether

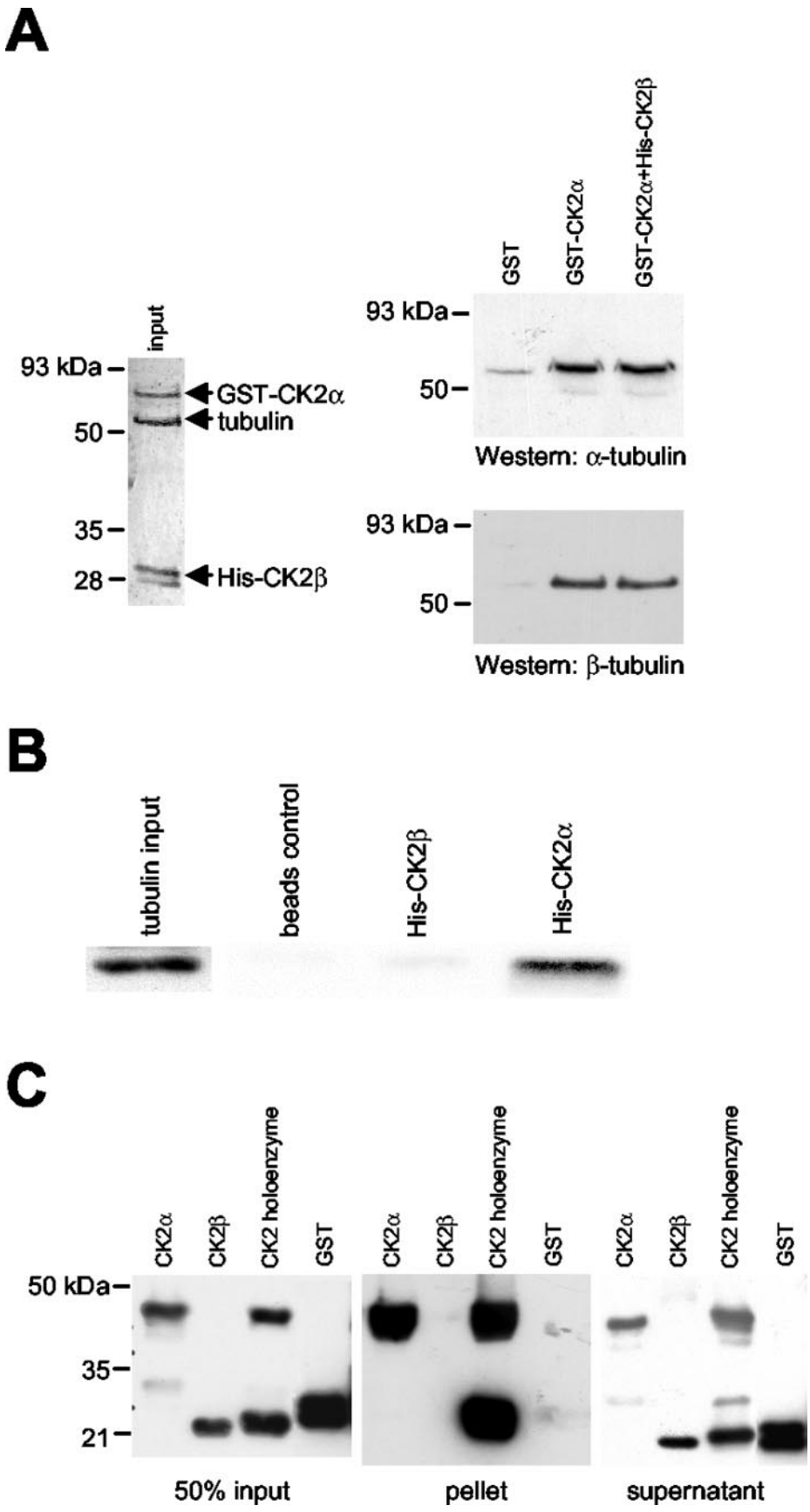

FIG. 1. **Microtubule association of CK2.** *A*, direct interaction of CK2 and tubulin heterodimers. 5 μg of GST, GST-CK2α, or a complex of GST-CK2α/His-CK2β were incubated with 2.5 μg of purified tubulin. The GST fusion proteins were then retrieved using GSH beads, and bound proteins were analyzed by immunoblotting with antibodies recognizing α- and β-tubulin. The protein input column was visualized by Coomassie Blue staining. *B*, CK2β does not interact physically with tubulin. 5 μg of His-CK2α or His-CK2β were incubated with 2.5 μg of purified tubulin. There was no His-CK2α or His-CK2β in the beads control sample. After pull-down with nickel-nitrilotriacetic acid beads, bound proteins were analyzed by anti-β-tubulin immunoblotting. *C*, direct association of CK2 with microtubules. 1 μg of GST, CK2α, CK2β, or CK2 holoenzyme was incubated with microtubules pre-assembled using taxol from 10 μg of purified tubulin. After precipitation of the microtubules, proteins in the supernatant and the microtubule pellet were analyzed by immunoblotting using an antibody mixture recognizing GST, CK2α, and CK2β.

they are resistant to cold treatment for microtubule disassembly (24). It has been found that CK2 is enriched in the cold-stable fraction of the microtubule preparation from rat brain (14). This observation, together with our findings that CK2 associates with microtubules to promote microtubule assembly, prompted us to explore the possibility that CK2 may contribute to the cold stability of microtubules. To test this likelihood, CK2-polymerized microtubules were incubated on ice, and the

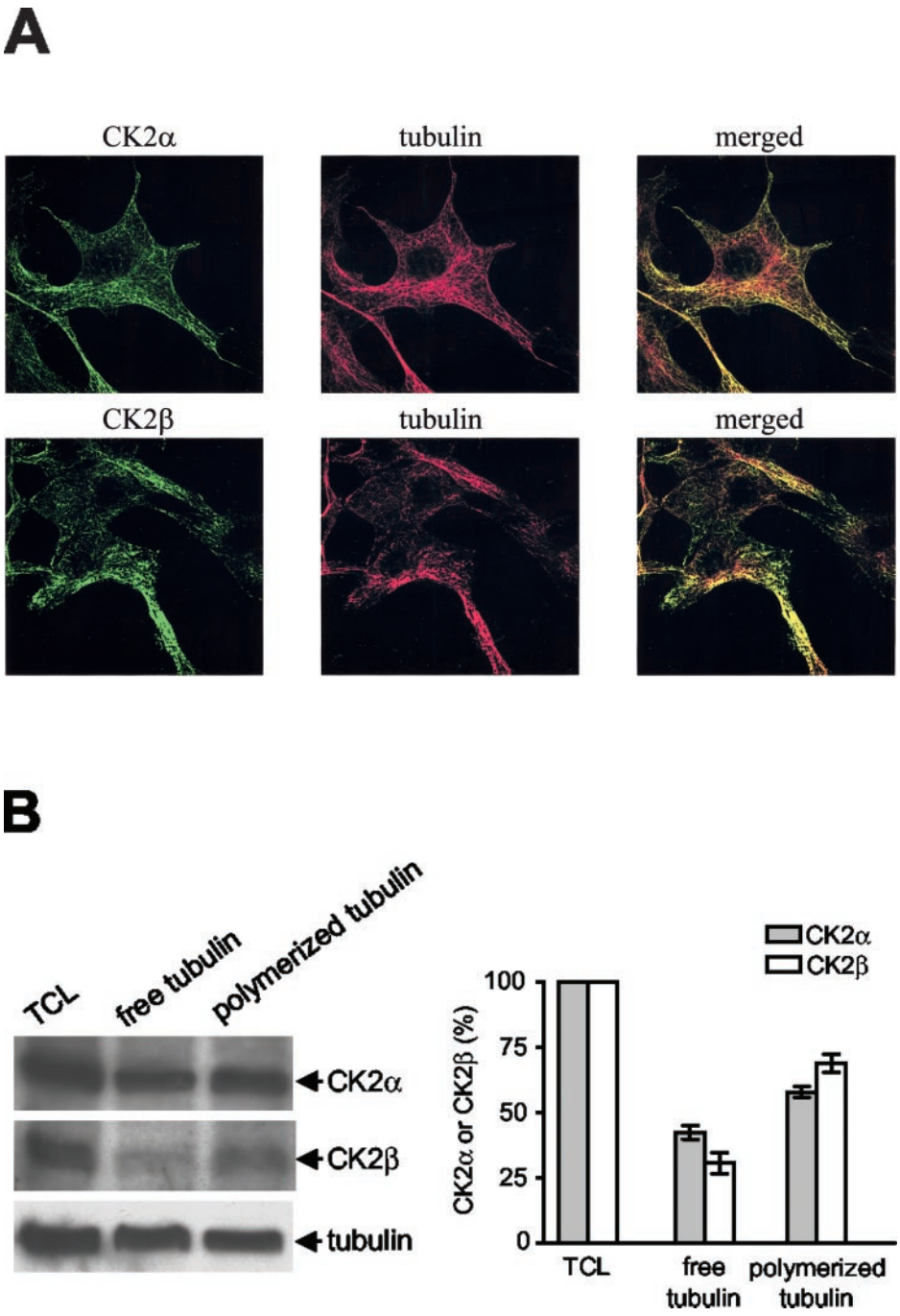

FIG. 2. **Cellular localization of CK2α and CK2β to microtubules.** *A*, COS-7 cells were immunostained for confocal microscopic analysis. *Top row*, double staining of CK2α and β-tubulin; *bottom row*, CK2β and β-tubulin. *B*, soluble tubulin heterodimers (free tubulin) and microtubules (polymerized tubulin) were differentially extracted from HeLa cells. Both fractions as well as the total cell lysate (*TCL*) were analyzed by immunoblotting using antibodies as indicated. The histogram shows the relative amounts of CK2α and CK2β in the free and polymerized tubulin fractions. These data are representative of three independent experiments.

turbidity change was monitored. As a comparison, tau-polymerized microtubules were treated under the same condition, given the fact that tau does not confer cold stability to microtubules (25). As expected, the tau-polymerized sample was depolymerized almost completely within a few minutes (Fig. 6). However, the turbidity of the CK2-polymerized sample was only marginally reduced even after a prolonged cold incubation (Fig. 6), indicating that CK2 functions to stabilize microtubules against cold-induced disassembly.

CK2 Stabilizes Microtubules in Vivo—To evaluate the role of CK2 in microtubule dynamics *in vivo*, we knocked down CK2α/α′ in HeLa cells by gene silencing using a siRNA duplex derived from the human sequence of CK2α/α′ (26, 27). As shown by the CK2α immunoblot, the introduction of CK2α/α′ siRNA into the cells led to a dramatic decrease of the CK2α/α′ proteins to a minimal cellular level (Fig. 7*A*). To assess the knockdown effect on microtubules, the amount of cellular microtubules (assembled insoluble tubulin polymers) was determined using the differential extraction method and immunoblotting (22). In addition, the integrity of the cellular microtubule network was examined by immunofluorescent staining and confocal microscopy. The knockdown of CK2α/α′ significantly reduced the cellular content of microtubules (Fig. 7, *A* and *B*), suggesting CK2 as one of the factors in stabilizing microtubules *in vivo*. We further assessed microtubule stability using colchicine, which is a microtubule-disrupting agent. When colchicine was applied at a low concentration (0.2 μM) onto the cells that were transfected with a scrambled siRNA sequence, most of the microtubule structure remained intact (Fig. 7, *A* and *B*). However, such a low dose of colchicine caused severe disruption of the microtubule structure in the CK2α/α′-depleted cells where the microtubule networks were collapsing toward the perinuclear membrane (Fig. 7*B*); almost negligible amount of microtubules was extracted from these cells (Fig.

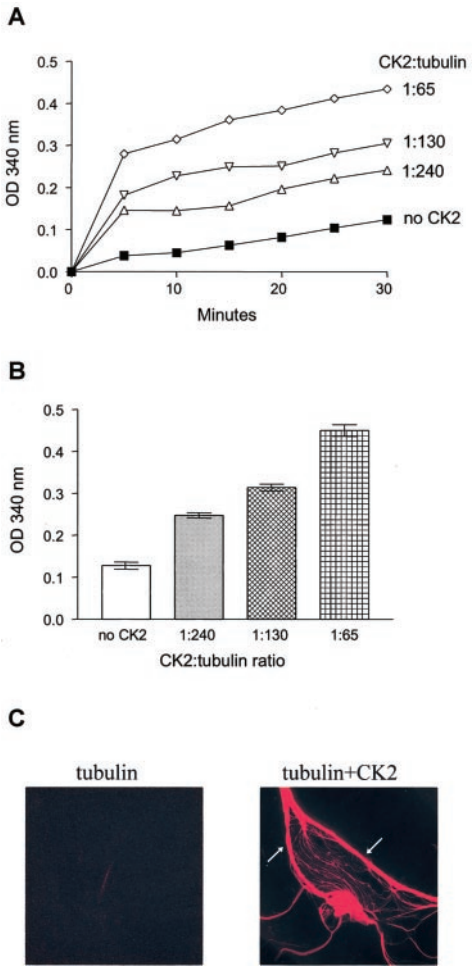

FIG. 3. **Effect of CK2 on microtubule assembly.** *A*, the turbidimetric assay of tubulin polymerization. Microtubule assembly from purified MAP-free tubulin was carried out in the presence of the CK2 holoenzyme at various concentrations (molar ratios to tubulin). The concentration of tubulin was constant in each assay at 2 mg/ml. *B*, histogram of the microtubule assembly at various amounts of CK2. The assembly assay was performed as described in *A* for 30 min. The data shown are representative of three separate experiments. *C*, fluorescent imaging of microtubules polymerized from a mixture of rhodamine-labeled and unlabeled tubulin (7:1). The tubulin concentration is 2 mg/ml, and the CK2 concentration is 62 μg/ml. The *arrows* point to microtubule bundles in the CK2-polymerized sample.

7*A*). Apparently, the removal of CK2α had a strong effect on cellular microtubule architecture, rendering it very unstable. As a result, it was readily destructed by colchicine at a very low concentration.

To further substantiate the microtubule stabilizing function of CK2, we tested whether microtubule stability could be restored by expression of chicken CK2α in endogenous CK2α/α'-depleted cells. As observed with the HeLa cells, knockdown of CK2α/α' in cultured human 293T fibroblasts using siRNA strongly destabilized the microtubule network, resulting in almost complete disruption of the microtubules by colchicine at 0.2 μM (Fig. 7*C*). When chicken CK2α was expressed in the 293T cells in which endogenous CK2α/α' was knocked down, the cellular microtubules completely retained their integrity against the colchicine-induced disruption (Fig. 7*C*). More interestingly, when the expression was performed using the kinase-inactive mutant CK2αK68A, it exhibited the same effect as wild-type CK2α in rescuing microtubules from colchicine treatment (Fig. 7*C*). These data demonstrate that CK2 is an important mediator of cellular microtubule stability and exerts its effect in a phosphorylation-independent manner.

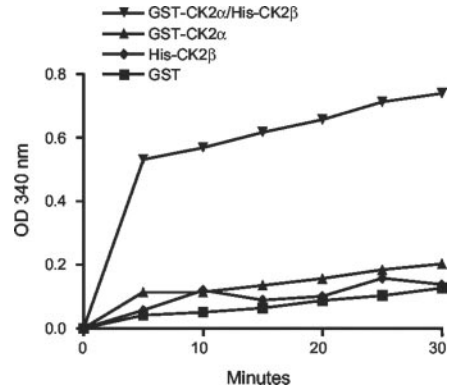

FIG. 4. **Microtubule assembly can be induced by the CK2 holoenzyme but not its individual subunits.** GST, GST-CK2α, and His-CK2β were applied as indicated at 0.1 mg/ml in the microtubule assembly assay. As a control, the CK2 holoenzyme reconstituted from the same amount of GST-CK2α and His-CK2β as described under "Experimental Procedures" was applied. Microtubule assembly was performed at 2 mg/ml tubulin as described under "Experimental Procedures."

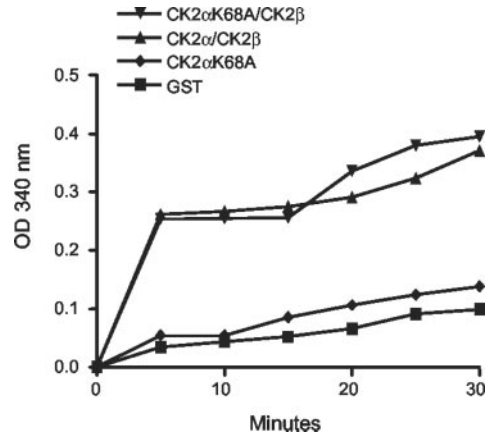

FIG. 5. **The kinase activity of CK2 is not required for its function to induce microtubule assembly.** The wild-type CK2 enzyme (GST-CK2α/His-CK2β) and the kinase-inactive enzyme (GST-CK2αK68A/His-CK2β) were applied as indicated at 0.1 mg/ml in the microtubule assembly assay. GST-CK2αK68A and GST were also tested at the same amount. Microtubule assembly was performed with 2 mg/ml tubulin as described under "Experimental Procedures."

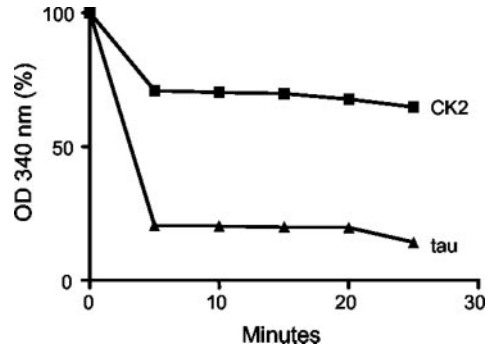

FIG. 6. **CK2 confers cold stability to microtubules.** Microtubules were polymerized with 0.05 mg/ml CK2 or 0.16 mg/ml tau protein for 30 min at 35 °C, where they attained similar turbidity measurements. The microtubule samples were then incubated on ice, and the turbidity measurement was begun. Absorbance was expressed as a percentage of the measurement when ice incubation was started.

DISCUSSION

Microtubules are a major cytoskeletal constituent in all eukaryotes. In living cells, the microtubule architecture is stabilized by structural MAPs, which associate with microtubules

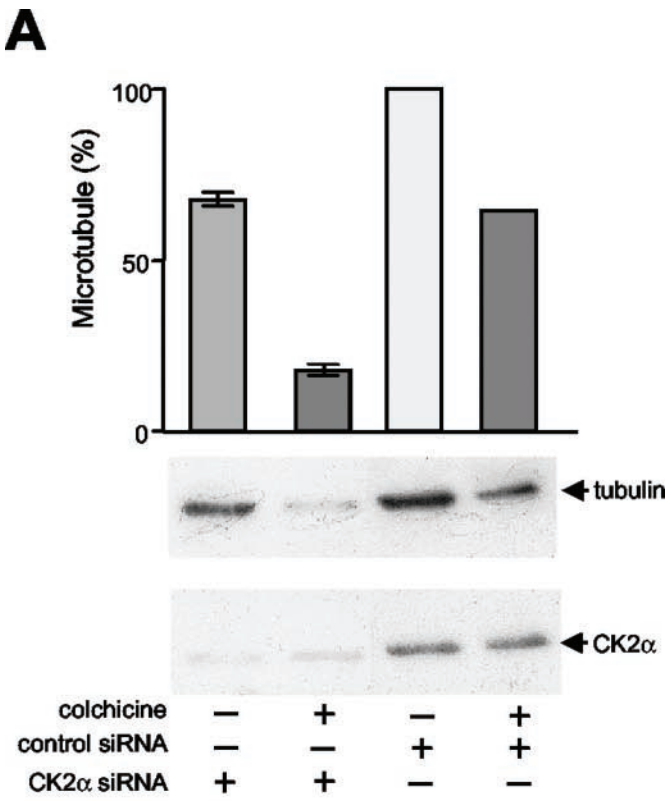

FIG. 7. **CK2 stabilizes microtubules *in vivo*.** *A*, HeLa cells were introduced with siRNA of human CK2α/α' or a scrambled sequence. Knockdown of CK2α was monitored by anti-CK2α immunoblotting. The cells were subsequently treated with 0.2 μM colchicine or its solvent. Tubulin in the form of polymers (microtubules) was extracted from the cells for β-tubulin immunoblotting. The histogram reflects the relative amounts of microtubules extracted from the cells as compared with the control, which is the sample transfected with the scrambled siRNA sequence and treated without colchicine. The data are representative of three separate experiments. *B*, cells in the experiments described in *A* were fixed and stained with the β-tubulin antibody for confocal microscopic imaging. *C*, expression of the wild-type or the kinase-inactive mutant of chicken CK2α restored microtubule stability against colchicine treatment in CK2α/α'-depleted cells. Prior to treatment with colchicine (0.2 μM), 293T cells were double transfected with siRNA of human CK2α/α' and one of the following expression constructs: chicken CK2α, the kinase-inactive mutant of chicken CK2α (CK2αK68A), or the empty vector. Expression of Myc-tagged chicken CK2α and CK2αK68A was detected by anti-Myc immunoblotting of the cell lysates. Microtubules were extracted using the differential extraction method (see "Experimental Procedures") for anti-β-tubulin immunoblotting. Representative results of three separate experiments are shown.

and promote *in vitro* microtubule assembly (28, 29). The evidence presented here identifies CK2 as a structural MAP that mediates microtubule dynamics. We have conducted experiments showing that CK2 is localized to and co-extracted with microtubules. The *in vitro* binding assays demonstrate the direct interaction of CK2 with microtubules as well as tubulin heterodimers, and the binding affinity is comparable with that of known MAPs. Microtubule binding sequences are often found in MAPs as repeated sequence stretches rich in basic amino acids. Although the sequence of CK2α contains some basic regions, it is not found to have any typical microtubule-binding motif in CK2α. Thus, the microtubule association of CK2α may suggest new microtubule-binding domains.

Structural MAPs such as MAP2 and tau are known to stimulate microtubule assembly from tubulin heterodimers. In our microtubule assembly assays, CK2 exhibited a potent activity of inducing microtubule assembly and bundling from purified tubulin. The physical association of CK2 to microtubules and tubulin heterodimers stimulates both the rate and the extent of microtubule growth. Although CK2α can bind microtubules, the microtubule assembling and stabilizing function is solely a property of the holoenzyme. In addition, CK2-polymerized microtubules display stability against cold treatment, suggesting that CK2 is a strong stabilizer of microtubules. Taken together with the observation that a substantial amount of CK2 exists in the cold-stable microtubules of rat brain (14), our findings suggest that CK2 is a new factor endowing the cold stability of microtubules. To date, the STOP proteins, double-cortin and BPAG1n3, are the only known MAPs that confer cold stability on microtubules (30–34).

Structural MAPs are known to contribute to microtubule stability and distribution within cells (35). The finding of CK2 as a structural MAP stimulated our interest in evaluating the regulatory role of CK2 *in vivo* in microtubule cytoskeleton. The knockdown of CK2α/α' from cells has a strong destabilizing effect on the microtubule architecture. As a result, the microtubule network is very vulnerable and can be readily destroyed by colchicine insult at 0.2 μM, whereas such a low concentration of colchicine does not have any significant effect on microtubules of cells with intact CK2. Thus, CK2 has an indispensable role in stabilizing cellular microtubules. This is substantiated by the introduction of chicken CK2α into the cells to compensate for the loss of endogenous CK2α/α'. The microtubule instability caused by the deficit of CK2α/α' can be rectified completely by the expression of chicken CK2α, which assures that CK2 is a vital structural MAP conferring microtubule stability *in vivo*. It is noteworthy that the removal of CK2α/α' did not cause severe microtubule disruption in the cells, possibly because of the existence of multiple MAPs other than CK2 in the cells, to support the microtubule network.

CK2 is a Ser/Thr protein kinase with a broad substrate spectrum that includes MAP1B and a neural-specific isoform of

β-tubulin. We examined whether the kinase activity of CK2 is involved in the microtubule assembly stimulated by CK2. Our *in vitro* assays of microtubule assembly using the kinase-inactive mutant of CK2 indicate that the microtubule-assembling activity of CK2 is independent of its kinase activity. This was corroborated by the experiment of expressing the kinase-inactive mutant of chicken CK2α in the CK2α/α′-knock-down cells, which completely compensated for the lost of endogenous CK2α/α′, rendering the microtubules resistant to colchicine attack. With these results, it becomes clear that CK2 imparts a direct regulation of microtubule organization through its physical association with microtubules but not through any enzymatic action. As a multifunctional enzyme, CK2 has been thought to execute its functions through its phosphorylation of a wide range of substrates. The results presented here reveal a novel CK2 function that is dissociated from its intrinsic kinase property.

It has been proposed that CK2 plays an important role in the maintenance of cell morphology and polarity. Depletion of the catalytic subunits of CK2 in neuroblastoma cells using an antisense approach blocks neuritogenesis (27, 36). Pertinent observations also came from yeasts, of which the temperature-sensitive mutants of CK2α and CK2β demonstrated their importance in cell morphogenesis (9, 37, 38). The function described here for CK2 in microtubule dynamics may provide a mechanistic explanation of its role in cell shape control.

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